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Molecular characterization of TGF-β mediated cancer cell proliferation

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I dedicate my thesis to my uncle late

Dr. U. V. Singh, my husband Shiv Kishor Singh

and my family

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SUMMARY

TGF-β inhibits epithelial cell growth through Smad-dependent induction of a cell cycle arrest at G1. During tumor progression, however, many tumor cells escape from TGF-β growth suppression due to either functional or genetic disruption of the Smad signaling pathway. In late tumor stages, TGF-β stimulates tumor cell proliferation through increased cell cycle transition. Although this cellular event is clearly established, the molecular mechanisms underlying this phenomenon remain unknown. Here, we show that TGF-β stimulation induces cancer cell proliferation via accelerated G1/S phase transition. We show that cell proliferation requires induction of the c-Myc oncogene, and this is paralleled by upregulation of D-type cyclins and their corresponding CdKs. TGF-β induces c-Myc expression on the level of promoter regulation through induction of the c-Myc/TIE element, which has previously been reported as the core element for repression of c-Myc in growth inhibited cells.

Mechanistically, TGF-β induces c-Myc promoter activation through the calcium responsive NFAT transcription factor family. We show that TGF-β induces expression and subsequent nuclear accumulation of NFATc1 and NFATc2 in cancer cells. NFAT proteins then bind to and displace Smad repressor complexes from the c-Myc/TIE to induce the c-Myc promoter transactivation and expression. c-Myc induction, on the other hand, is essential for subsequent cyclin upregulation and stimulation of G1/S phase transition in cancer. Thus, this study uncovers a key signaling and transcription pathway in TGF-β growth stimulation and identifies the NFAT transcription factor family as essential mediators of this function. From the medical point of view this study not only help to better understand the mechanisms underlying TGF-β growth promotion in cancer, but also provide a platform for novel options in the treatment of pancreatic cancer. In fact, recent approaches led to the identification of novel and interesting

molecules that target the NFAT pathway more specifically in tumor cells. Future studies will show whether specific targeting of the NFAT system in tumor cells is a promising new strategy in the treatment of pancreatic cancer and in particular in those with high levels of TGF-β.

ZUSAMMENFASSUNG

In normalen epithelialen Zellen und in frühen Tumorstadien bewirkt TGF-β eine starke Tumorsuppression, die v.a. auf der Smad vermittelten Induktion eines Zellzyklus-Arrests beruht. Während der Tumorprogression verlieren jedoch sehr viele Tumorzellen ihre Fähigkeit auf einen TGF-β Stimulus mit einer Wachstumshemmung zu antworten. Dies kann sowohl in genetischen Alterationen der TGF-β/Smad Signalkaskade, als auch in funktionellen oder epigenetischen Veränderungen dieses Signalweges oder wichtiger Interaktionspartner begründet sein. In späten Tumorstadien stimuliert TGF-β dann sogar das Wachstum epithelialer Zellen, obgleich die zugrundeliegenden molekularen Mechanismen nur unzureichend verstanden sind. Im Rahmen der vorliegenden Untersuchung konnten wir zeigen, dass TGF-β das Wachstum von Pankreaskarzinomzellen durch Induktion einer gesteigerten Zellzyklus-Progression stimuliert, was mit einer Zunahme der Expression von D-Typ Zyklinen und ihrer Aktivatoren einhergeht. Von zentraler Bedeutung ist hierbei die Promotoraktivierung und Induktion des mitogenen Transkriptionsfaktors c-Myc.

Wir konnten zeigen, dass die Induktion von c-Myc nicht nur essentiell für die konsekutive Zellzyklus-Progression ist, sondern auch von einer vorangehenden Induktion der NFAT Transkriptionsfaktoren abhängt. TGF-β induziert Smad-unabhängig die Expression und nukleäre Akkumulation von NFATc1 und NFATc2, welche anschließend am TIE-Element des proximalen c-Myc Promotors binden. Das TIE Element wurde in früheren Studien als „TGF-β-inhibitory element“ beschrieben und ist für die TGF-β vermittelte Repression des c-Myc Promotors und der Vermittlung einer Wachstumshemmung in epithelialen Zellen wichtig. In Pankreaskarzinomzellen führt die Bindung von NFAT am TIE Element des c-Myc

Promotors zur Verdrängung DNA-bindender Smad3-Repressorkomplexe und anschließenden Induktion der c-Myc Transkription, G1-Progression und Proliferation. Dieser neue Mechanismus der TGF-β-Wachstumsregulation ist von zentraler Bedeutung für das phänotypische Verhalten von Pankreaskarzinomzellen und streng abhängig von der sequentiellen Induktion der pro-proliferativen Transkriptionsfaktoren NFAT und c-Myc. Diese Arbeit verdeutlicht auch die Rolle Smad-unabhängiger transkriptionell regulatierter Mechanismen im Pankreaskarzinom und untermauert eine wichtige Funktion der NFAT Transkriptionsfaktoren in der Karzinogenese epithelialer Tumoren. Letztlich liefert unsere Untersuchung erste Hinweise auf ein therapeutisches Potenzial der gezielten NFAT Hemmung in der Behandlung des Pankreaskarzinoms.

1 INTRODUCTION

1.1 THE PLURIPOTENCY OF TRANSFORMING GROWTH FACTOR- BETA

The transforming growth factor beta (TGF-β) signalling pathway is a key player in metazoan biology, and its misregulation can result in tumor development (Levy et al., 2006). The regulatory cytokine TGF-β exerts tumor-suppressive effects that cancer cell must elude for malignant evolution (Cui et al., 1996; Massague et al., 2000). Yet, paradoxically, TGF-β also modulates processes such as cell invasion and proliferation, immune regulation, and microenvironment modification that cancer cells may exploit to their advantage (Massague et al., 2008). Consequently, the output of a TGF-β response is highly contextual throughout development, across different tissues, and also in cancer. The mechanistic basis and clinical relevance of TGF-β's role in cancer is becoming increasingly clear, paving the way for a better understanding of the complexity and therapeutic potential of this pathway.

1.2 TGF-BETA SIGNALLING ACTIVATION

TGF-β signals through two classes of receptors, the TGF-β type I receptor (TβRI) and the TGF-β type II receptor (TβRII). Endoglin and betaglican, also called accessory receptors, bind to TGF-β with low affinity and present it to the TβRI and TβRII (Shi et al., 2003). Type I and II receptors are serine/threonine kinase receptors that form a heterodimeric complex upon TGF-β binding. TGF-β interacts with the ectodomain of the TβRII and allows the subsequent incorporation of the TβRI generating a ligand-receptor complex formed by a ligand dimer. The TβRII

appears to be a constitutively active kinase that, when the ligand–receptor complex is formed, phosphorylates a characteristic SGSGSG sequence, called the GS domain, present in the type I receptor. Phosphorylation of the TβRI GS domain leads to the activation of its kinase and turns the GS region into a Smad binding site. Once activated by the TβRII, the TβRI initiates activation of distinct intracellular signalling pathways, most notably the Smad signalling cascade (Seoane et al., 2006).

1.3 THE SMAD SIGNALLING PATHWAY IN NORMAL AND MALIGNANT EPITHELIAL CELLS

Smad proteins are central mediators of TGF-β signalling. They can be classified into three subtypes, e.g. receptor-regulated Smads (R-Smads), common-partner Smads (Co-Smads), and inhibitory Smads (I-Smads) (Miyazono et al., 2000). Of the eight different Smad proteins in mammals, Smad2 and Smad3 serve as R-Smads for TGF-β signalling pathways, Smad4 acts as a Co-Smad (Moustakas et al., 2001) and Smad7 functions as an I-Smad for TGF-β signalling (Nakao et al., 1997). Smad proteins have conserved N- and C-terminal regions, termed the MH1 and MH2 domains, respectively. The MH1 and MH2 domains are bridged by linker regions. In all three subtypes of Smads, MH2 domains are highly conserved (Miyazono et al., 2000). In contrast, MH1 domains are conserved in R-Smads and Co-Smads, whereas the N-terminal regions of I-Smads are highly divergent from those of R- and Co-Smads (Nakayama et al., 2001).

R-Smads are anchored to the plasma membrane through various molecules, among which Smad anchor for receptor activation (SARA) has been most extensively studied (Tsukazaki et al., 1998). SARA has a FYVE domain, which is responsible for binding to phosphatidyl inositol-3-phosphate in the plasma membrane. SARA preferentially binds to the MH2 domains of Smad2 and Smad3,

but not to those of other Smads. SARA forms a dimer in cells, and anchors two molecules of Smad2/3 to the plasma membrane, which may be important for efficient activation of R-Smads by the hetero-tetrameric TβRII and TβRI complexes (Wu et al., 2000). The activated TβR-I kinase phosphorylates the last two serine residues at the C-terminal Ser-Ser-X-Ser motif of R-Smads. R-Smads then form heteromeric complexes with Co-Smad through their MH2 domains, and translocate into the nucleus (Shi et al., 2003). Although the exact structures of the R-Smad/Co-Smad heteromers have not been fully determined, a heterotrimer composed of two molecules of R-Smads and one molecule of Co-Smad, or a hetero-dimer composed of one molecule each of R-Smad and Co-Smad, has been proposed (Itoh et al., 2000). R-Smads and Co-Smads shuttle between the nucleus and cytoplasm.

Nuclear localization signals (NLSs) in the MH1 domains of R-Smads play pivotal roles in translocation of Smads into the nucleus, whereas nuclear export signals (NESs) in the MH2 domains of R-Smads and those in the linker region of Co-Smads are responsible for nuclear export of the complex (Reguly et al., 2003). In the nucleus, the R-Smad-Co-Smad heteromers interact with various transcription factors and transcriptional co-activators or co-repressors, resulting in transduction of a wide variety of intracellular signals in target cells (Wrana et al., 2000). R-Smads and Co-Smads also directly bind to specific DNA sequences, although with relatively low affinities. Thus, Smads and other transcription factors cooperatively regulate transcription of target genes through binding to their promoters. Transcriptional co-activators, including p300, CBP, and P/CAF, contain histone acetyl transferase (HAT) domains (Janknecht et al., 1998; Feng et al., 1998; Itoh et al., 2000). Through acetylation of histones and probably other proteins, these transcriptional co-activators help Smads activate the transcription of target genes (Massagué et al., 2000; Derynck et al., 1998).

1.4 GENETIC ALTERATIONS OF THE SMAD SIGNALLING PATHWAY

Genetic alterations of this simple TGF-β signalling pathway are frequently found in somatic and heritable disorders as well as in various tumor entities (Gold et al., 1999). Inactivating mutations of the type-II receptor, for instance, have been reported in colorectal, gastric and endometrial carcinomas and in association with a more aggressive phenotype (Markowitz et al., 1995; Teicher et al., 2001). In addition, more than half of pancreatic carcinomas and approximately one third of colorectal carcinomas harbor inactivating mutations of the Smad4 gene based on homozygous deletion or intragenic mutation (Moore et al., 2001; Takagi et al., 1996). In many cases, genetic loss of Smad4 causes disrupted nuclear-cytoplasmic shuttling of Smad complexes and thus renders tumor cells insensitive to nearly all TGF-β regulated transcriptional responses, including those associated with growth inhibition (Miyaki et al., 2003). Genetic alterations of the Smad4 gene are also found in familial juvenile polyposis, an autosomal dominant disease characterized by predisposition to gastrointestinal polyps and cancer (Huang et al., 2009; Chow et al., 2005). In this syndrome, polyps are formed by inactivation of the Smad4 gene through germline mutation and loss of the unaffected wildtype allele (Chow et al., 2005).

Together, disruption of TGF-β-Smad signalling through inactivating mutations are commonly observed in different malignancies and is closely associated with the loss of tumor suppression by TGF-β. Importantly, however, signal transduction and target gene transcription by the Smads is not only affected by genetic alterations within the pathway, but also through signalling crosstalk interactions (Bierie et al., 2006). In fact, at one level or another, essentially all major tumor suppressor and oncogenic signalling pathways can converge on Smads as a mode for signal integration and thus can positively or negatively influence Smad-mediated

transcription. Inputs by other pathways occur at the level of receptor activation, R-Smad complex formation with Co-Smad4, or at the level of the nuclear translocation of pre-formed R-Smad/Co-Smad complexes.

Crosstalk between Smads and distinct mitogen-activated protein kinases (MAP kinases) such as extracellular signal regulated kinases (Erk) 1 and 2 and the stress-activated protein kinases, Jun N-terminal kinase (JNK) and p38, are of particular interest in carcinogenesis (Hocevar et al., 1999). For instance, mutational activation of MAP kinase pathway is frequently found in human carcinomas, most notably *via* the activation of oncogenic Ras, an upstream activator of the proliferative Raf-Mek-Erk signalling cascade, which is an early event in many tumors, such as pancreatic cancer (Giehl et al., 2000). Persistent activation of MAP kinase pathways by oncogenic Ras inhibits Smad signalling through Erk mediated phosphorylation of MAP kinase sites within the linker region of Smad2 and Smad3, thereby blocking the complex formation with Smad4 (Kretzschmar et al., 1999).

The linker region is a critical phosphorylation site for several kinase regulated signalling pathways. For instance, Smad proteins contain multiple phosphorylation sites for Ca2+/calmodulin dependent protein kinase II (CamKII) and protein kinase C (PKC), which phosphorylate Smad2 and Smad3 and thereby inhibit either the nuclear translocation or the DNA binding of Smads (Wicks et al., 2000). In contrast, signalling crosstalk with JNK or MEKK1, an upstream activator of the JNK pathway, has been shown to facilitate the nuclear accumulation of Smad complexes and thereby accelerate Smad-signalling in cancer cells.

Together, the Smad signalling transduction pathway is commonly altered by either genetic alterations or crosstalk interactions with cascades that play important functions in gene regulation during carcinogenesis.

1.5 SMAD-INDEPENDENT SIGNALLING IN NORMAL AND MALIGNANT EPITHELIAL CELLS

Although Smad signalling is considered the central TGF-β signalling pathway, many studies have revealed additional effectors in downstream signalling that fundamentally affect the transcriptional response to TGF-β (Moustakas et al., 2001; Warna et al., 2000). Based on their mode of actions, the non-Smad proteins are categorized in three different groups: (i) non-Smad signalling pathways that directly modify Smad function; (ii) non-Smad proteins whose function is directly modulated by Smads and which transmit signals to other pathways; and (iii) non-Smad proteins that directly interact with or become phosphorylated by TGF-β receptors but do not necessarily affect the function of Smads.

Best studied non-Smad proteins are members of the MAPK family (Yue et al., 2000). TGF-β-induced activation of Erk, JNK and p38 MAPK kinase pathways causes a broad spectrum of cellular responses including cell proliferation, apoptosis and differentiation (Lehmann et al., 2000). Depending on cellular activation, induction of MAPKs can occur in a Smad-dependent or Smad-independent fashion (Engel et al., 1999). Smad-independent activation of p38 MAPK, for instance, has recently been demonstrated in Smad4-deficient cells and in cells with mutated TGF-β type-I receptors, defective in Smad activation (Yu et al., 2002). Following TGF-β stimulation, MAPKs can then regulate gene transcription through either direct activation of specific transcription factors, such as Elk or AP-1, or through crosstalk interaction with the Smads resulting in positive or negative effects on TGF-β-mediated transcription.

The group of receptor-induced non-Smads comprises important regulatory FKBP12 (Wang et al., 1996), TRIP-1 and the B subunit of the protein phosphatase 2A, PP2A (Griswold-Prenner et al., 1998; Petritsch et al., 2000) each of which have

been identified by their specific interaction with the TGF-β type-I receptor. PP2A consists of a catalytic C domain and two regulatory subunits, termed subunits A and B. The regulatory B subunit is one example among other WD40 proteins, that can modulate TGF-β-induced transcription (STRIP1, STRAP) or links TGF-β to modulate translation (Strip1, EIF2alpha). Upon TGF-β-binding, the B subunit of PP2A associates with the activated type-I receptor, enhances PP2A activity and allows the recruitment of p70S6K, a kinase with a key role in translational control and cell-cycle progression. Another important family of TGF-β effector proteins is members of the Rho-like GTPases which have emerged as alternative components of intracellular signalling pathway originating from the TGF-β receptor. There is much evidence that the Rho family of proteins mediates many cytoskeletal effects as well as cell motility, and maintaining focal contacts and contractile stress fibers.

It has been reported that RhoA plays a central role in TGF-β-induced epithelial to mesenchymal transdifferentiation, EMT, (Bhowmick et al., 2001) and that TGF-β can stimulate cell motility and cytoskeletal organization via activation of RhoA. Some of these effects might be regulated on the transcriptional level, some other occur indirectly and through interaction with other signalling cascades. In human hepatoma cells, TGF-β activates protein kinase C (PKC) (Miyazaki et al., 2004; Sakaguchi et al., 2004). PKC then phosphorylates the regulatory protein S100C/A11, which translocates to the nucleus to recruit Sp1 to the promoters of the p15 and p21 genes. This pathway is very similar to the parallel Smad pathway, which also induces these genes through interactions with Sp1 (Feng et al., 2000; Pardali et al., 2000). Together, many non-Smad signalling proteins modulate the activity of the Smad pathway and therefore, can potentially affect gene regulation by TGF-β in both normal and malignant cells.

1.6 TGF-BETA GROWTH CONTROL IN NORMAL AND MALIGNANT CELLS

Most pertinent to our understanding the role of TGF-β in carcinoma development is the fact that TGF-β is a potent inducer of growth inhibition in several cell types, including epithelial cells. One key event that leads to TGF-β induced growth arrest is the induction of expression of the CdK inhibitors $p15^{INK4B}$ and/or $p21^{CIP1}$, depending on the cell type (Massague 2008). The inhibitor $p21^{CIP1}$ interacts with complexes of CdK2 and cyclin A or cyclin E and thereby inhibits CdK2 activity, preventing progression of the cell cycle. By contrast, $p15^{INK4B}$ interacts with and inactivates CdK4 and CdK6, or associates with cyclin D complexes of CdK4 or CdK6 (Warner et al., 1999). The latter interaction not only inactivates the catalytic activity of these CdKs but also displaces $p21^{CIP1}$ or the related $p27^{KIP1}$ from these complexes, allowing them to bind to and inactivate the CdK2 complexes with cyclin A and E. Induction of $p15^{INK4B}$ or $p21^{CIP1}$ expression in response to TGF-β is brought about by Smad-mediated transcriptional activation.

In contrast to many TGF-β responses that are mediated by Smad3 and Smad4, a heteromeric complex of Smad2, Smad3 and Smad4 induces transcription by interacting with Sp1 at the $p15^{INK4B}$ or the $p21^{CIP1}$ promoter (Feng et al., 2000; Li et al., 1995). Consequently, the Smad complex recruits the coactivator CBP/p300 into the complex and strongly potentiates the transcriptional activity of Sp1, which activates transcription of the $p15^{INK4B}$ or $p21^{CIP1}$ genes. Additional mechanisms also contribute to TGF-β–mediated growth arrest, again depending on the cell type. Most important, TGF-β inhibits the expression of the c-Myc oncogene. High levels of c-Myc render the cells resistant to the growth inhibitory activity of TGF-β, and downregulation of c-Myc is required for the induction of $p15^{INK4B}$ and $p21^{CIP1}$ expression (Seoane et al., 2001). The interaction of c-Myc in a complex at the $p15^{INK4B}$ promoter correlates with transcriptional repression; TGF-β induced down

regulation of c-Myc thus allows the repression of cell by TGF-β–induced transcription of $p15^{INK4B}$ through Smads. Chen and coworkers have recently identified a TGF-β inhibitory element of the c-myc promoter which is responsible for TGF-β-induced repression of the c-Myc (Chen et al., 2002). This group has further demonstrated that TGF-β-induced c-Myc repression can be mediated by interaction of Smad3, p107 and members of the E2F transcription factor family. E2F4 and E2F5 associate with p107 and assemble a complex with Smad3 in the cytoplasm that translocates into the nucleus following TGF-β receptor activation (Chen et al., 2002). Increasing evidence also suggests a role for the TGF-β-inducible non-Smad transcription factors in TGF-β-induced cell growth inhibition.

Most recently, our laboratory has explored a novel mechanism in c-Myc repression that was mediated through a Smad3-TIEG2 complex. Following TGF-β stimulation, nuclear TIEG2 interacts with Smad3 *via* its DNA-binding zinc-finger domain and co-operatively represses myc-transcription from the TGF-β inhibitory element (TIE) of the human c-myc promoter. We demonstrated that the TIE element comprises a TIEG2-binding site and an adjacent Smad binding element, both of which were integral in conferring full promoter repression by TGF-β (Ellenrieder et al., 2002). Mutational inactivation of either the TIEG- or the Smad binding element significantly lowered TGF-β-responsiveness of the TIE element. Moreover, disruption of KLF11-Smad3 interaction or artifical knockdown of endogenous KLF11 expression strongly diminished Smad3-TIE binding, caused loss of c-Myc repression and rendered epithelial cells less sensitive for TGF-β-induced cell growth inhibition (Ellenrieder et al., 2008). Together, new data clearly demonstrate that Smad and TIEG2 effector proteins synergize in TGF-β-induced cell growth inhibition through co-operative repression of the c-Myc proto-oncogene.

1.7 MECHANISMS UNDERLYING THE DUAL ROLE OF TGF-BETA DURING TUMORIGENESIS

Similar to its effects on normal epithelial cells, TGF-β inhibits tumor growth at early tumor stages. As the tumor progresses, however, genetic disturbances of the TGF-β-signalling pathway may occur and render the tumor cells insensitive to TGF-β-induced growth inhibition. It is abundantly clear that loss-of-function mutation of TGF-β signalling components is one route towards loss of growth control in cancer (Massague et al., 2008). On the other, it became also clear, that many tumor cells become refractile to the growth inhibitory effects of TGF-β despite lack of genetic alterations of the TGF-β signalling pathway. These tumor cells are of particular interest in tumor research as they frequently respond to TGF-β with increased migration and proliferation, and severe phenotypic changes described as an epithelial-to-mesenchymal transdifferentiation (Seoane et al., 2008; Muraoka-Cook et al., 2005).

During tumor progression, crosstalk interactions between Smads and oncogenic signalling pathways occur at different cellular levels and in all cellular compartments (Ellenrieder et al., 2001). For instance, altered activation of oncogenic signalling cascades such as the proliferative Ras-Raf-Erk MAPK pathway dramatically affects both the constellation and the activation status of Smad-interacting transcription factors in the nucleus and this influences the transcriptional reponse to TGF-β in tumor cells. A good example came from our laboratory showing that Smad3/TIEG2 mediated repression of c-myc is blocked in pancreatic cancer cells with oncogenic Ras mutations. We have further characterized this phenomenon and identified a novel mechanism involved in the loss of TGF-β-growth inhibition. We could show that in pancreatic cancer cells with oncogenic Ras mutation, hypersensitive Erk MAPK phosphorylates TIEG2 at four serine/threonine sites within the linker region between the R1 and R2 repression

domains and subsequently inhibits binding of the Sin3A corepressor (Ellenrieder et al., 2002, Ellenrieder et al., 2004). Erk MAPK induced disruption of Sin3A corepressor binding results in loss of TIEG2-mediated c-myc repression, an effect that can be prevented by introduction of Erk-insensitive TIEG2 mutants. Thus, results from our laboratory together with observations from other groups contributed to a better understanding of how inactivation of Smad partnering transcription factors affect Smad-mediated transcription and anti-proliferation. Together, these studies suggested that Smad inactivation is a key process during in the loss of growth suppression by TGF-β in cancer (Massague et al 2000).

Moreover, crosstalk with oncogenic signalling pathways might also play roles in TGF-β-stimulated tumor progression. Prominent examples are again mitogen-activated protein (MAP) kinase pathways, which are commonly observed in tumor cells, and the activation of which is required for TGF-β-induced expression of extracellular matrix degrading proteases. TGF-β often promotes tumor progression through induction of an epithelial-mesenchymal transdifferentiation of tumor cells (Oft et al., 1996, Ellenrieder et al., 2001). EMT is a characteristical feature of highly invasive tumor cells and result from interactions of Smads with oncogenic signalling pathways members. Several signalling pathways have already been identified to play role in TGF-β-induced EMT. Of particular relevance are again crosstalks with Jagged/Notch signalling PI3K-AKT signalling, RhoA, Rac1 and p38MAPK (Zavadil et al., 2004, Bakin et al., 2002; Horowitz et al., 2004). The requirement for an intact Smad signalling was demonstrated by using a mutant TGβRI construct that failed to bind Smads, but can still activate MAPK pathways.
Despite observations that Ras exerts inactivating phosphorylation on Smads, in case of EMT synergy between TGF-β and Ras-Raf-MAPK pathways to promote EMT was reported and observed by our group (Ellenrieder et al., 2001). Especially in pancreatic tumors a high number of activating mutations in Ki-Ras oncogene can be observed. Together with an intact TGF-β signalling pathway, this might account

for the migratory capacities of pancreatic adenocarcinoma in many patients. However, in contrast to the mechanisms responsible for EMT, migration and invasion, there is only very limited information about those mechanisms that mediate cell proliferation by TGF-β.

In fact, it is still unclear whether TGF-β utilizes the Smad pathway to mediate growth in advanced tumor stages or whether alternative signalling and transcription pathways are more important during gene regulation in cancer growth.

2 AIMS OF THE STUDY

The role of TGF-β in cancer biology is complex and involves aspects of tumor suppression as well as tumor promotion. Overexpression of TGF-β expression in the tumor cells ecpecially in pancreatic cancer have seen and which creates a tumor micro-environment that stimulates both tumor growth and metastasis. The diametrically opposed roles of TGF-β as a potent tumor suppressor and as a tumor promoter has gained widespread acceptance in recent years.

In contrast to the mechanisms underlying tumor cell migration and invasion, the signalling and transcription pathways involved in the TGF-β switch from a growth suppressor to a strong promoter of tumor cell growth are only marginally understood. We therefore conducted this study to analyse how TGF-β stimulates pancreatic cancer cells to proliferate.

In particular we addressed the following specific questions:

1. Does TGF-β induce cell proliferation in cancer?

2. Does TGF-β stimulate cancer cell proliferation on the level of gene transcription?

3. Which signalling and transcriptional pathways confer cancer growth promotion by TGF-β?

3. MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Cell Lines

1. HaCaT (Human keratinocyte cell line)
2. Panc1 (human pancreactic cancer cell line)
3. 8988t (human pancreactic cancer cell line)

3.1.2 Chemical reagents and general materials

Reagent	**Source**
Cell strainer (70 µM)	Falcon
2 ml cryotubes	Greiner bio-one
Disposable needles, cuvettes & syringes	Greiner bio-one
Glasswares	Schott
Nitrocellulose membrane	Schleicher & Schuell
Polypropylene tubes	Greiner bio-one
Parafilm	Greiner bio-one
Pipette tips	Eppendorf
Pipettes	Sarstedt
Röntgen film (13x18 cm, BioMax)	Amersham
Sterile filters (0.2 µM/ 0.45 µM)	Schleicher
Tissue culture plates	Greiner bio-one
Tissue culture flask (50, 250, 500 ml)	Greiner bio-one
Tissue culture dish (60 mm, 90 mm)	Greiner bio-one
Tubes (1.5 & 2 ml) Sarstedt	Eppendorf

Whatmann paper	Schleicher & Schuell
12 well cell culture plates	Nunc
6 & 24 well cell culture plates	Nunc
6 mm Petri dishes	Nunc

3.1.3 Chemical reagents

Chemicals	**Source**
Acetone	Carl Roth
Acrylamid solution	Carl Roth
Agar	Carl Roth
Agarose	Sigma-Aldrich
Ampicillin	Sigma
APS Merck	Eurolab
β-Mercaptoethanol	Carl Roth
BioRad protein assay	BioRad
Bromophenol blue	Sigma
Bovine serum albumin (BSA)	Sigma
Calcium chloride [$CaCl_2$]	Carl Roth
Cyclosporin A [CsA]	Novartis Pharma
dNTPs	MBI-Fermentas
DTT	Carl Roth
ECL chemiluminiscence	Amersham
EDTA	Carl Roth
EGTA	Sigma-Aldrich
Ethanol	Carl Roth
Ethidium Bromide [EtBr]	Sigma-Aldrich
Formaldehyde	Carl Roth
Forskolin	Calbiochem

ECL chemiluminescence kit	Pierce & GE healthcare
Gene Ruler™ 100bp ladder	MBI Fermentas
Glycerin (87%)	Carl Roth
Glycine	Carl Roth
Hepes	Carl Roth
Hydrochloric acid [HCl]	Merck Eurolab
Isoamylalcohol	Carl Roth
Isopropanol	Carl Roth
Leupeptin hydrochloride	Roche
Lithium chloride	Sigma-Aldrich
Milk powder	Sigma
Magnesium acetate	Sigma-Aldrich
Magnesium chloride	Carl Roth
Magnesium sulfate	Carl Roth
Manganese chloride	Fluka
Methanol	Carl Roth
Phenol	Carl Roth
PMSF	Roche
Proteinase K	Sigma-Aldrich
Protein A sepharose	Upstate
Protein G sepharose	Upstate
Polyacrylamide	Applichem
Ponceau Red	Sigma-Aldrich
Potassium acetate	Carl Roth
Potassium chloride	Sigma-Aldrich
Potassium dihydrogen phosphate	Sigma-Aldrich
Potassium hydrogen phosphate	Sigma-Aldrich
Potassium hydroxide	Carl Roth
Propidiumiodide	Sigma-Aldrich

Protease inhibitor tablet (complete mini)	Roche
Sodium azide	Sigma
Sodium citrate	Sigma
Sodium acetate	Merck Eurolab
Sodium carbonate	Carl Roth
Sodium chloride	Carl Roth
Sodium fluoride	Sigma-Aldrich
Sodium hydrogen phosphate	Merck Eurolab
Sodium hydroxide	Carl Roth
SDS	Carl Roth
TEMED	Carl Roth
TGF-β	Tebu-Bio

3.1.4 Transfection reagents

Reagents	**Source**
Transfast for plasmid transfection	Promega
Transmessenger for SiRNA transfection	Qiagen
SilentFect for SiRNA transfection	Bio-Rad

3.1.5 Radioactive chemical

Reagents	**Source**
[methyl-^{3}H] thymidin 50Ci/mmol 1mCi/ml	Amersham Biosciences

3.1.6 Instruments

Hardware	Manufacturer
Autoclave	Stiefenhofer
Bacterial shaker	New Brunswick Scientific
Balance machine	Sartorius
Cold centrifuge	Eppendorf
FACS Calibur	Becton Dickinson
Gel camera	Stratagene
Heating blocks	Eppendorf
Ice machines	Genheimer
Laminar hoods	Heraeus
Liquid nitrogen tank	Tec-lab
Luminometer	Berthold
Microliter pipettes	Eppendorf
Microcentrifuge	Eppendorf
Microscope	Ziess
pH meter	Ingold
Refrigerators (-20°C; -80°C)	Privileg and Bosch
Shaking incubator	Eppendorf
Spectrophotometer	Amersham Pharmacia
Vortexer	Eppendorf
Waterbath	Eppendorf
Western blot apparatus	Hoefer
Real-time PCR machine	ABI Prism 7000
DL Ready^{TM}Luminometer	Berthold Technologies

3.1.7 Kits

Dual Luciferase Reporter Assay Kit	Promega
RNeasy Mini Kit	Qiagen
Plasmid-DNA isolation kit (Maxi)	Qiagen
PCR purification kit	Qiagen

3.1.8 PCR reagents

Reagents	**Source**
dNTPs	MBI Fermentas
$MgCl_2$	MBI Fermentas
10X Buffer	MBI Fermentas
Taq polymerase	MBI Fermentas

3.1.9 Real-time PCR primers for cDNA

c-Myc

5’ GCTCCTGGCAAAAGGTCAGA 3 ’

5’ CAGTGGGCTGTGAGGAGGTT 3’

NFATc1

5’ TGCAAGCCGAATTCTCTGG 3’

5’ GGGAAGGTAGGTGAAACGCTG 3’

NFATc2

5’ GTTCCTACCCCACAGTCATTCAG 3’

5’ CCCGCAGGTAATACTTCCTTTTG 3’

cyclophilin A

5’ CACCGTGTTCTTCGACATCA 3’

5’ AGCA TTTGCCATGGACAAGAT 3’

XS-13

5’ GTCGGAGGAGTCGGACGA 3’

5’ GCCTTTATTTCCTTGTTTTGCAAA 3’

3.1.10 Real-time primers for ChIP

c-Myc-4

5’ AGGGCTTCTCAGAGGCTTG 3’

5’ TGCCTCTCGCTGGAATTACT 3’

c-Myc 17

5’ GAGGGATCGCGCTGAGTAT 3’

5’ GCTGGAATTACTACAGCGAGTTAGA 3’

siRNAs:

NFATc2

#2 - 5’ GCUGAUGAGCGGAUCCUUATT 3’

#3 - 5’ CCAUUAAACAGGAGCAGAATT 3’

NFATc1

#1 - 5’ CCAUUAAACAGGAGCAGAATT 3’

#2 - 5’ GCUGAUGAGCGGAUCCUUATT 3’

Smad3

#1 - 5’ GCAUCCGCUGUUCCAGUGGUTT 3’

#2 - 5’ ACACUGGAACAGCGGAUGCTT 3’

c-Myc

#1 - 5' GGAACGAGCUAAAACGGAGTT 3’

#2 - 5’ CUCCGUUUUAGCUCGUUCCT 3’

3.1.11 Expression vectors

Vectors	Backbone	Tags
wt-NFATc2	pcDNA3.1	HA
wt-NFATc1	pREP4	-
NFATc2 promoter	pcDNA3.1	Luc
NFATc1 promoter	pREP4	Luc
c-Myc-I	pBV-Luc	Luc
c-Myc-II	pBV-Luc	Luc
c-Myc-III	pBV-Luc	Luc
c-Myc-IV	pBV-Luc	Luc
c-Myc-V	pBV-Luc	Luc
c-Myc-VI	pBV-Luc	Luc
c-myc-pGL3-A	pGL3-Enhancer	Luc
c-myc-pGL3-B	pGL3-Enhancer	Luc
c-myc-pGL3-C	pGL3-Enhancer	Luc
c-myc-pGL3-D	pGL3-Enhancer	Luc
c-myc-pGL3-E	pGL3-Enhancer	Luc
c-myc-pGL3-F	pGL3-Enhancer	Luc
c-myc-pGL3-NFATmut	pGL3-Enhancer	Luc

3.1.12 List of antibodies for Western blot analysis

Antibodies	Dilutions	Source
Mouse anti-mouse NFATc1	1:1,000	Abcam
Mouse anti-mouse NFATc2	1:500	Santa Cruz
Mouse anti-mouse Smad3	1:1,000	Abcam
Mouse anti-mouse Smad2/3	1:500	B.D Biosciences
Rabbit anti-rabbit pSmad3	1:1,000	Cell Signalling
Mouse anti-mouse Smad4	1:1,000	B.D Biosciences
Mouse anti-mouse c-Myc	1:500	Santa Cruz
Mouse anti-mouse CyclinD1	1:1,000	Cell Signalling
Mouse anti-mouse CyclinD3	1:1,000	Cell Signalling
Mouse anti-mouse CdK4	1:1,000	Cell Signalling
Mouse anti-mouse CdK6	1:1,000	Cell Signalling
Mouse anti-mouse β-actin	1:10,000	Sigma
Rabbit anti-mouse Lamin A/C	1:1,000	Santa Cruz

3.1.13 List of antibodies for ChIP analysis

Antibodies	Amount	Source
Rabbit anti-human NFATc1	4 µg	ImmunoGlobe
Rabbit anti-human NFATc2	4 µg	ImmunoGlobe
Mouse anti-mouse SMAD3	4 µg	Abcam

3.2 SOLUTIONS AND BUFFERS

3.2.1 Mediums and buffer solutions

All chemicals of molecular biology research grade were procured from respective manufacturers and all solutions were prepared using pure distilled (Milli-Q grade) autoclaved water. Wherever necessary, solutions were sterile filtered or autoclaved.

3.2.1.1 Cell biological

PBS (Phosphate buffered saline)

8 g	NaCl
0.2 g	KCl
1.44 g	Na_2HPO_4
0.24 g	KH_2PO_4

Dissolve in 800 ml dH_2O, adjust pH to 7.4 with HCL

Volume adjust to 1 L, autoclave and stored at RT

Reagents	**Source**
DMEM medium	Invitrogen
L-Glutamin (0.07%)	GIBCO
Na-Pyruvat (1 mM)	GIBCO
β-Mercaptoethanol (0.05 mM)	GIBCO
MEM (non-essential amino acids) (1%)	GIBCO
Penicillin (100 U/ml)	Grunthal
Streptomycin (100 U/ml)	Fatol

FCS (fetal calf serum) (1-10%)	GIBCO

3.2.1.2 Biochemical

SDS-PAGE

Composition of SDS-Polyacrylamide gels:

Resolving Gel

Component volumes (ml) per 10 ml gel mix for 10% to 15%

H_2O 3.3 - 4.6 ml

30% acrylamide mix 2.7 ml - 4.0 ml

1.5 M Tris (pH 8.8) 2.5 ml

10% SDS 0.1 ml

10% ammonium persulfate 0.1 ml

TEMED 0.004 ml

Stacking Gel:

Component volumes (ml) per 3 ml gel mix for 10% to 15%

H_2O 2.1 ml

30% acrylamide mix 0.5 ml

1.0 M Tris (pH 6.8) 0.38 ml

10% SDS 0.03 ml

10% ammonium persulfate 0.03 ml

TEMED 0.003 ml

Protein Loading Buffer (1X SDS gel loading buffer)

50 mM Tris-Cl (pH 6.8)

100 mM Dithiothreitol

2% SDS

0.1% Bromophenol blue

10% Glycerol

Gel Running Buffer: (10X)

144.13 g Glycine

30.3 g Tris

100 ml 10% SDS

Volume adjusted to 1 L with dH_2O

Protein transfer buffer: (10X)

145 g Glycine

29 g Tris

volume adjusted to 1 L with dH_2O

Blocking Solution

5% (w/v) nonfat dried milk in TBS/0.1% Tween

Agarose gel electrophoresis

TBE buffer (1x):

10.8 g	Tris
5.5 g	Boric acid
0.37 g	EDTA
add	1 L H_2O

DNA Loading Buffer:

0.25% Bromophenol blue

0.25% Xylene Cyanol FF

30% Glycerol in water

1% Agarose Gel:

1.5 g Agarose

150 ml 1x TBE buffer

boil and add 4 µl Ethidium bromide (10 mg/ml)

Cell extract preparation

Whole cell extract (WCE) buffer:

5mM Tris HCL, pH7.4

150 mM NaCl

1 mM EDTA

1% Triton X-100

Protease inhibitor cocktail was added immediately before use.

Cellular Fractionation Buffer

For cytoplasmic protein:

10 mM Hepes, pH 7.9

10 mM KCl

0.1 mM EDTA

0.1 mM EGTA

0.1 M DTT

For nuclear protein:

20 mM Hepes, pH 7.9

0.4 M NaCl

1 mM EDTA

1 mM EGTA

0.1 M DTT

Protease inhibitor cocktail was added immediately before use

Cell lysis buffer for IP:

5 mM Tris HCL, pH7.4

150 mM NaCl

1 mM EDTA

1% Triton X-100

(10X) wash buffer for IP

0.5 M Tris HCL, pH 7.4, 1.5 M NaCl

Protease inhibitor cocktail was added immediately before use

3.2.1.3 Reagents for ChIP analysis

Lysis buffer I (hypotonic lysis buffer):

5 mM PIPES, pH 8

85 mM KCl

0.5% NP40

Protease inhibitor cocktail was added immediately before use.

Lysis buffer II:

10 mM Tris-HCL, pH 7.4

150 mM NaCl

1% (v/v) NP40

1 mM EDTA

1% sodium deoxycholate (fume hood)

0.1% SDS

Protease inhibitor cocktail was added immediately before use.

Wash buffer I (mixed micelle buffer):

20 mM Tris, pH 8.1
150 mM NaCl
2 mM EDTA
0.1% SDS
0.1% Triton X-100

Wash buffer II (buffer 500):

20 mM Tris, pH 8.1
500 mM NaCl
2mM EDTA
0.1% SDS
0.1% Triton X-100

Wash buffer III (LiCl detergent buffer):

10 mM Tris, pH 8.1
250 mM LiCl
1% NP40
1% sodium deoxycholate (Fume hood)
1mM EDTA

TE buffer:

20mM Tris, pH 8.0
1mM EDTA, pH 8.0

Elution buffer:

500µl 20% SDS

1ml 1M Na_2CO_3

H_2O upto 10 ml

Always prepare fresh elution buffer

3.3 METHODS

3.3.1 Preparation of competent cells ($CaCl_2$ method)

Inoculate an overnight pre-culture from a single colony on a prestreaked plate (from glycerol stock) in 2 ml LB or 2X TY media by incubation at 37°C and shaking to aerate. On the second day, inoculate 1 ml of the pre-culture in 100 ml fresh media and grow the culture at 37°C until OD at wavelength 600 nm. Cool down the culture on ice for at least 15 min. The following procedures should be carried out at 4°C in pre-cooled sterile tubes. Harvest the cells in a centrifuge at 5000 rpm for 5 min, and discard the supernatant. Resuspend the bacterial pellets thoroughly in a small volume of ice-cold 100 mM $CaCl_2$. Dilute the suspension with the $CaCl_2$ solution to a final volume of 30-40 ml, and leave on ice for 25 min with occasional shaking. Spin down the cells as before, discard the supernatant carefully and resuspend the pellet in 5 ml glycerol/$CaCl_2$. The suspension can be aliquoted in 100 to 400 µl and stored at –80°C. The transformation efficiency of the bacteria prepared by this method should reach at least $5X10^6$cfu/mkg of plasmid DNA.

3.3.2 Transformation of competent bacteria

Thaw the competent bacteria on ice. Add ligated DNA or purified plasmid-DNA to 100 µl suspension of competent cells in a cold 1.5 ml microfuge tube. Mix carefully and keep on ice for 30 min. Heat-shock the bacteria at 42°C for 40 sec, chill on ice for 2 min and add 500 µl antibiotic-free LB medium, and shake at 37°C for 1 hr. Selection of transformed bacteria is done by plating aliquots of the bacterial suspension on agar plates containing respective antibiotics. A single colony can then be picked, expanded in LB medium and used for DNA preparation.

3.3.3 Bacterial manipulation

Plasmid transformed bacteria are selected on LB plates with the appropriate antibiotic for 24 hr. For overnight mini cultures, single colonies are picked and inoculated into LB medium with antibiotic and shaken overnight at 37°C. This pre-culture is then used for preparing frozen glycerol cultures, plasmid DNA or protein purification. For storage of bacteria, a glycerol stock culture is prepared by growing bacteria to an OD of 0.8 at a wavelength of 600 nm in culture medium. 500 µl bacterial cultures has to be added to 500 µl 80% glycerol and then mixed thoroughly in a small 1.5 ml tube. This stock solution is subsequently frozen at –80°C. To inoculate an overnight culture again, bacteria are taken and held at room temperature (RT) until surface is thawed. A small amount of cells is picked, mixed into 2-5 ml culture medium and left to grow for 12-16 hr at 37°C in a bacterial culture shaker.

Plasmid	Antibiotics used
c-Myc-I	Amp
c-Myc-II	Amp
c-Myc-III	Amp
c-Myc-IV	Amp
c-myc-V	Amp
c-myc Del. VI	Amp
c-Myc pGL3A	Amp
c-myc-pGL3-B	Amp
c-myc-pGL3-C	Amp
c-myc-pGL3-D	Amp
c-myc-pGL3-E	Amp
c-myc-pGL3-F	Amp

c-myc-TIE-NFATm	Amp
NFATc2 wt	Amp
NFATc1 wt	Amp
Smad3 wt	Amp

3.4 EXPERIMENTAL PROCEDURES

3.4.1 Cells and transfection

Panc-1 (ATCC, CRL-1469), 8988t (DSMZ, ACC 162) and HaCaT (CLS #300493) cells were maintained in DMEM (Invitrogen Life Technologies, Karlsruhe, Germany) supplemented with 10% FCS. Expression and reporter promoter plasmids were transfected at 70% cell confluence using TransFast (Promega, Madison, WI). Short interfering RNA (siRNA) was transfected using TransmessengerTM reagent (Qiagen, Hilden, Germany) or SiLentfect (from bio-Rad), according to the manufacturer's instructions and cells were treated with 10 ng/ml TGF-β and harvested at indicated time points.

3.2.4 Preparation of whole protein extract from mammalian cells

Cells were washed with ice cold PBS and then scraped in whole cell extract buffer. Then transferred them into 1.5 ml tubes. Kept on ice for 30 min to 1 h. Cells were disrupted by two times freezing and thawing on dry ice or passing the cell suspension through 26 gauge needle 10 times and incubated for further 10 min. The cell suspension was centrifuged at 15,000 rpm for 30 min at 4°C and supernatant was saved as whole cell extract in another 1.5 ml

tube, which was stored for future use at -20°C. The protein concentration of the supernatant was determined by Bio-Rad protein assay.

3.2.5 Preparation of nuclear and cytoplasmic protein extracts from mammalian cells

Cells were washed and scraped in cold PBS. Transferred them into 1.5 ml tube and centrifuge at 1500 rpm for 5 min at 4°C to remove the supernatant. The pellet was resuspended in 200 µl to 1 ml of extraction buffer A (100 µl per $1x10^7$ cells) and incubated for 20-30 min at 4°C or on ice. Extraction buffer A is a low salt buffer (indicates that DTT and PMSF were added to buffer A), which allowed the cells to swell. To destroy the swollen cells, the solution was passed 10 times through 1 ml syringe with 26 gauge needle and centrifuged at 3,600 rpm for 20 min at 4°C. The supernatant contained cytosolic fraction and the pellet, which appeared transparent, containing nuclear fraction. The supernatant was transferred to a fresh 1.5 ml tube and kept on ice. The pellet was resuspended with 200 µl extraction buffer C (leupeptin was added in addition to DTT and PMSF) by pipetting and vigorously mixing with force, followed by vortexing the nuclear extract vigorously for 30 min and incubate on ice for 30 min. Now the suspension was centrifuged at 15,000 rpm for 30 min at 4°C and supernatant containing nuclear proteins was frozen in –20°C. The protein concentration of the supernatant was determined by Bio-Rad protein assay.

3.2.6 Protein determination

The Bio-Rad protein assay is based on the observation that when Coomassie brilliant blue G-250 binds to the protein, the absorbency maximum shifts from 450 nm to 595 nm. Equal volumes of cell lysate containing 1-20 µg of protein was added to diluted dye reagent and mixed well (1:5 dilution of dye reagent concentrate in dH_2O). After 5-10 min, the absorption at wavelength at 595 nm was measured versus reagent blank (which contains only the lysis buffer).

3.2.7 SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gels were prepared in 8 cm x 10 cm x 1.5 mm mini gel format according to the standard Laemmli method. Separating or lower gel mix was prepared according to the volume required, poured in the gel apparatus, overlaid gently with 0.1% SDS and before the separating gel polymerized, immediately poured the stacking gel, the comb was inserted and allowed to polymerize at RT for 1 h to 2 h. Requisite concentrations of protein samples were mixed with 4x Laemmli buffer and denatured by heating at 95°C for 5 min, loaded in the wells, (one well was loaded with protein marker) of polymerized gel and electrophoresed at constant current initially at 120 V and when the marker start separating current increased up to 160 V per gel in 1X SDS-PAGE running buffer.

3.2.8 Western blotting

SDS-PAGE gel was electrotransferred onto nitrocellulose membrane at 300-400 mA for 90 min at 4°C. The air dried membrane was incubated in a

blocking solution (5% fat free milk in 1X TBS-Tween) for 1 h at RT. Membrane was directly incubated in primary antibodies against cyclinD1, CDK4, CDK6, GSK3β, HA, Lamin a/c, HDM2, p53, NFATc2, Ubiquitin and β-actin antibodies over night at 4°C. After incubation, the membrane was washed in 1X TBS-Tween for 3 X 10 min each. Now membrane was incubated in secondary antibodies conjugated with peroxidase against mouse or rabbit antibody for 1-2 h at room temperature and washed in 1 X TBS-Tween for 3 X 10 min each. Proteins were visualized and developed with ECL developing solution according to the manufacturer's instructions.

3.2.9 siRNA transfection

Small interfering RNA (siRNA) was transfected using the Transmessenger^{TM} reagent (Qiagen) or SiLentfect (Bio-Rad) according to the manufacturer´s instructions. The specific siRNAs were purchased from Ambion Applied Biosystems (Austin, TX) with the following sequences: NFATc1 #2 5' GGACUCCAAGGUCAUUUUCTT 3'; NFATc2 #3 5' CCAUUAAACAGGAGCAGAATT 3'; #2 5' GCUGAUGAGCGGAUCCUUATT 3'; Smad3 #1 5' GCAUCCGCUGUUCCAGUGGUTT 3'; #2 5' ACACUGGAACAGCGGAUGCTT 3'; c-Myc #1 5' GGAACGAGCUAAAACGGAGTT 3'; #2 5 'CUCCGUUUUAGCUCGUUCCT 3'. As a negative control, the silencer negative-control from Ambion was used.

3.2.10 Proliferation assay and cell cycle analysis

Panc-1, 8988t or HaCaT cells were seeded in 12-well plates and cultured in medium containing 10% FCS until attachment. After attachment, cells were starved for 24 hr in serum free medium and either transfected with siRNA or treated with TGF-β (10 ng/µl) for indicated time periods. [^{3}H] thymidine

(0.5uCi/well) was added during the last 6 hr of incubation. Incorporated [^{3}H] thymidine was quantified as described previously. For statistical analysis, Students t-test was used and $p < 0.05$ was considered significant. Cell cycle analysis was performed by flow cytometry. Cells were treated with 10 ng/µl TGF-β for 0 hr, 18 hr, 24 hr and 48 hr, then trypsinized, washed with PBS and fixed in 70% ethanol. After washing with PBS, cells were incubated with 20 µg/ml RNase, DNase-free water with 50 µg/ml propidium iodide for 3 hr at RT under light protection. The DNA content of 10^6 cells was analyzed on a Becton Dickinson FACS Calibur flow cytometer (San Jose, CA). The fractions of cells in the G0/G1, S and G2/M phases were calculated using Cell Quest software from Becton Dickinson (Topsham, ME).

3.2.11 RT-PCR

RNA was extracted using the RNeasy Mini Kit (Qiagen) and first-strand cDNA was synthesized from 1 µg total RNA using random primers and the Omniscriptfirst-strand synthesis kit (Qiagen) according to the manufacturer's instructions. The qRT-PCR was performed using a 7500 Fast-Real-Time-PCR-System from Applied Biosystems (Foster City, MA). Specific primer pairs were designed with the PrimerExpress 3.0 (Applied Biosystems, Wellesley, MA) as followed: cyclophilin A for 5' CACCGTGTTCTTCGACATCA 3', rev 5' AGCA TTTGCCATGGACAAGAT 3'; c-Myc for 5' GCTCCTGGCAAAAGGTCAGA 3', rev 5' CAGTGGGCTGTGAGGAGGTT 3'.

3.2.12 DNA pull-down

Panc1 and 8988t cells were treated with TGF-β (10 ng/µl) for the indicated time periods. In total, 100 µg of nuclear protein per sample were incubated

for 3 hr with 1 µg of biotinylated double-stranded oligonucleotides containing the GGAAA consensus NFAT-binding sequence of the wild-type TIE element (wt TIE), the wild type TIE element (TIE-wt, _92 to _63 relative to the c-myc P2 transcription start site; 50- 5' TTCTCAGAGGCT TGGCGGGAAAAAGAACGG 3' -30 and 5' CCGTTCTTTTTCCCGCCAAGCCTCTGAGAA 3') or the NFAT mutant TIE sequence (TIE-M1; 50- 5' TTCTCAGAGGCTTGGCGGGCCCAAGAACGG 3'-30 and 5' CCGTTCTTGGGCCCGCCAAGCCTCTGAGAA 3'). DNA–protein complexes were collected by precipitation with streptavidin–agarose beads (Sigma-Aldrich) for 1 hr, washed twice with lysis buffer including proteinase and phosphatase inhibitors and subjected to SDS-PAGE analysis.

3.2.13 Chromatin Immunoprecipitation Analysis (ChIP)

ChIP assays were performed in Panc-1 and 8988t cells treated with TGF-β (10 ng/µl) for the indicated time periods. Cells were cross-linked with 1% formaldehyde for 10 min at 37°C, harvested in SDS lysis buffer (Upstate Biotechnology), and DNA was shredded to fragments of 500 bp by sonification. Antibodies against NFATc2, NFATc1 or Smad3 were added to each aliquot of pre-cleared chromatin and incubated over night. Protein G agarose beads were added and incubated for 1.5 hr at 4°C. After reversing the cross-links, DNA was isolated and used for PCR reactions. Specific primer pairs were designed with the Primer Express 3.0 as followed: c-Myc primer 4 for.- 5' AGGGCTTCTCAGAGGCTTG 3', rev.- 5' TGCCTCTCGCTGGAATTACT 3' and c-Myc primer 17 for- 5' GAGGGATCGCGCTGAGTAT 3' rev - 5' GCTGGAATTACTACAGCGAGTTAGA 3' for quantitative PCR amplifying the TIE element.

3.2.14 Luciferase Reporter Assays

For luciferase reporter gene assays, 10^6 cells were seeded into 12-well tissue culture dishes and transfected after 24 hr with the indicated constructs. Treatment with TGF-β (10 ng/µl) as maintained 24 hr after transfection for the indicated time periods. Luciferase assays were performed with a Lumat LB 9501 luminometer (Berthold Technologies) and the Dual-Luciferase® -Reporter Assay System (Promega). Firefly luciferase values were normalized to Renilla luciferase activity and were either expressed as relative luciferase activity (RLA) or as mean 'fold induction' with respect to empty vector control. Mean values are displayed +/-standard deviations.

3.2.15 Statistical analysis

Each experiment was reproduced at least three times. Values are expressed as the mean ± SD of triplicate measurements unless otherwise stated. Student's paired *t*-test was used to analyze differences between the sample of interest and its control. Time courses and dose responses were compared by multiple measurements ANOVA and corrected by student-Newman–Keul's test for differences between groups. A *p* value of less than 0.05 was considered statistically significant.

4 RESULTS

4.1 TGF-BETA PROMOTES CELL PROLIFERATION VIA G1/S PHASE PROGRESSION IN CANCER CELLS

TGF-β signalling is known for its functional diversity in malignant and non-malignant cells. In order to test the effect of TGF-β on cancer cell growth, we performed proliferation assays in the pancreatic cancer cell lines 8988t and Panc-1. Therefore, serum starved cells were stimulated with TGF-β 10 ng/ml for indicated time periods. Cell proliferation was assessed by incorporation of [^{3}H] thymidine. TGF-β stimulation caused a significant increase in cell proliferation of Panc-1 and 8988t cells. Induction of cell proliferation occurred in a time dependent manner and became significant after 48hr. This effect was independent of Smad4 signalling (Figure 1) because one of the tested cell line has mutations in the Smad4 gene (8988t (Elsässer et al., 1992)). As TGF-β is known for its growth inhibitory effects in non-malignant epithelial cells, we tested HaCaT keratinocytes, which are sufficiently growth inhibited by TGF-β (Figure 2).

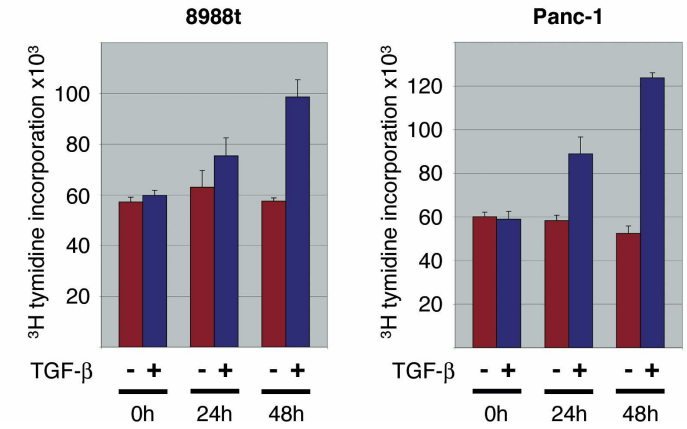

Figure 1) TGF-β promotes cell proliferation. Proliferation assays demonstrated the increased growth of Panc-1 and 8988t in response to TGF-β. Cells were left in serum free medium untreated

or treated with 10ng/µl TGF-β over the indicated time periods, 0 hr, 24 hr and 48 hr. Cell proliferation was assessed by incorporation of [^{3}H] thymidine in all the cells. Data are representative of triplicate experiments and are displayed as bars ± SD.

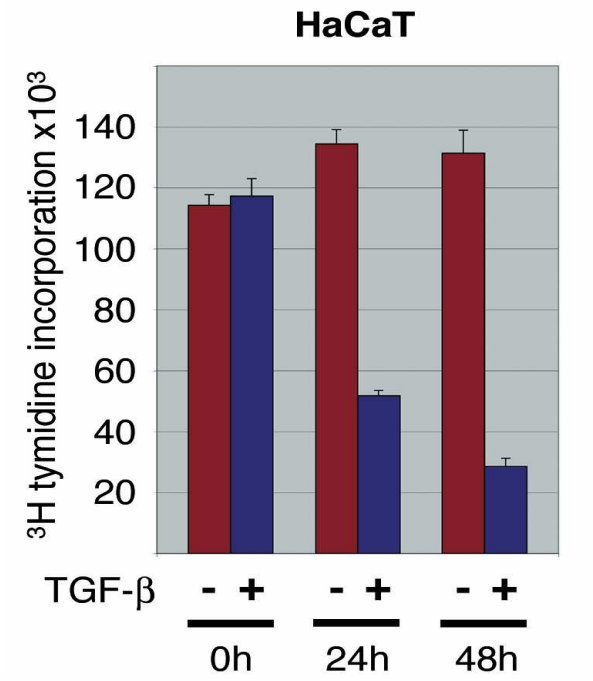

Figure 2) TGF-β inhibits cell proliferation in keratinocytes. The influence of TGF-β 10 ng/ml on growth inhibition in HaCaT cells was assessed by incorporation of [^{3}H] thymidine. Cells were kept in serum free medium with TGF-β (blue bars) or without TGF-β (red bars) for 24 and 48 hr. Data are representative of triplicate experiments and are displayed as bars ± SD.

Flow cytometry analysis revealed that the growth promoting effect of TGF-β in 8988t and Panc-1 cells resulted from increased cell cycle progression, as evidenced by the shift of cells from G1 to S and G2 phases (Figure 3). Moreover, cell cycle progression in growth promoted cells was reflected by increased expression of D-type cyclins (cyclin D1 and cyclin D3) and their corresponding kinases CdK4 and CdK6 (Figure 4), which are known to be required for cell proliferation. TGF-β stimulation induced the expression of cyclin D1 and cyclin D3 and their related kinases CdK4 and CdK6 in a time dependent manner.

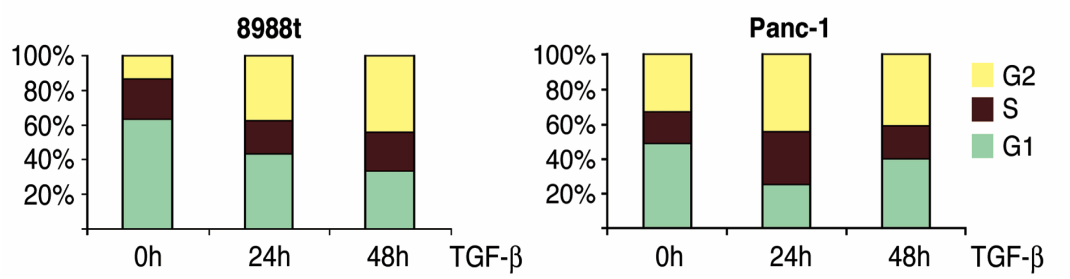

Figure 3) TGF-β induces G1/S phase transition in pancreatic cancer cells. Flow cytometry analysis was performed after propidium iodide (PI) staining in response to 10 ng/µl TGF-β treatment for 24 hr and 48 hr. Cell cycle stages are illustrated in different colors: G2 (yellow), S (brown), and G1 (green). Bars indicate mean values ± SD of three independent experiments.

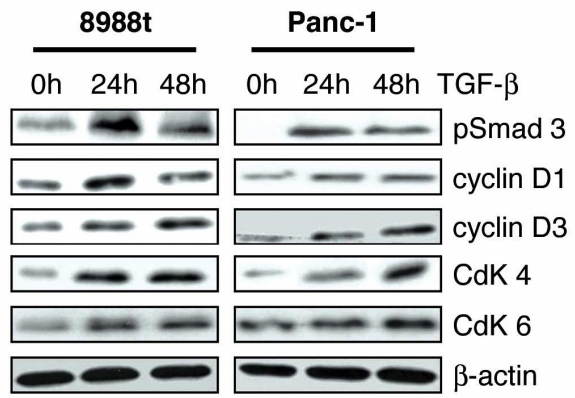

Figure 4) TGF-β induces G1/S phase transition via D-type cyclins. Western blot analysis examined the effect of TGF-β on the expression of cell cycle regulatory genes in growth promoted cell lines. Panc-1 and 8988t cells were incubated for 0 hr, 24 hr, and 48 hr in the presence (+) or absence (-) of TGF-β as indicated. Total cell lysates were prepared and then visualized by immunoblotting by using antibodies for phosphorylation of Smad3 at C-terminal, cyclinD1, cyclinD3, CdK4 and CdK6. Immunoblotting for β-actin served as a marker for total cell lysates.

4.2 c-MYC IS REQUIRED FOR TGF-BETA INDUCED CANCER CELL PROLIFERATION

To explore the TGF- β induced cell proliferation in more detail, we examined the c-Myc expression in cancer cells after TGF-β stimulation. C-Myc acts as transcriptional activator of growth and proliferation in response to growth stimuli. Interestingly, we found that TGF-β stimulation induced the c-Myc expression on both mRNA (Figure 5A) and protein level (Figure 5B) in growth promoted Panc-1 and 8988t cells. As expected and known before, TGF-β reduced the c-Myc expression on both mRNA (Figure 6A) and protein level (Figure 6B) in growth inhibited HaCaT cells.

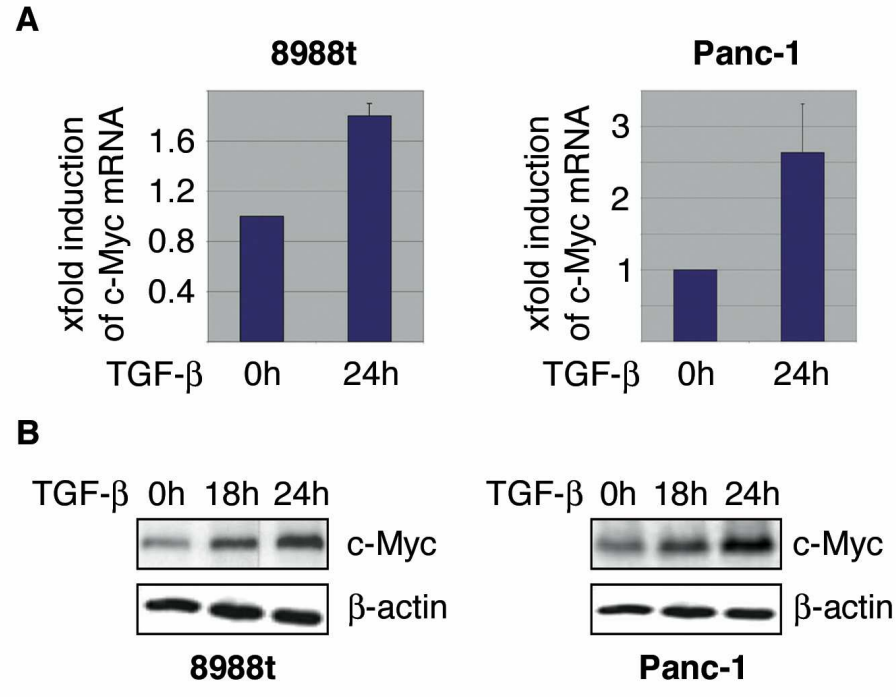

Figure 5) TGF-β induces c-Myc expression on mRNA and protein level in cancer cells. (A) TGF-β mediated induction of c-Myc mRNA expression was analyzed by RT-PCR in Panc-1 and 8988t cells. Serum starved cells were left untreated or treated with 10 ng/µl TGF-β for 24 hr before RNA extraction. mRNA expression levels were calculated relative to basal mRNA expression, which were arbitrarily set to 1 for each experiment, and expressed as fold induction. (B) Induction of c-Myc was

confirmed on protein level in Panc-1 and 8988t cells after stimulation with 10 ng/µl TGF-β for 18 hr and 24 hr. Total cell lysates were prepared and analyzed for c-Myc protein content by using anti-c-Myc antibodies. Protein loading was controlled using β-actin antibodies.

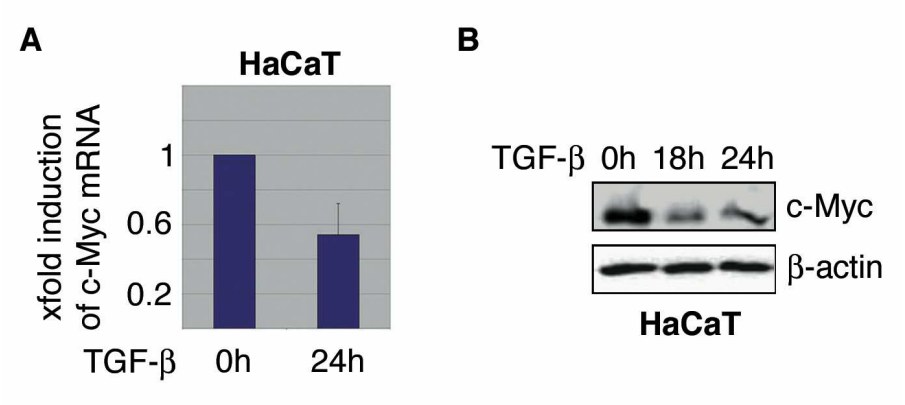

Figure 6) TGF-β down regulates c-Myc expression on mRNA and protein level in HaCaT cells. (A) Real time-PCR analysis to demonstrate c-Myc mRNA and protein expression in HaCaT cells upon TGF-β stimulation. Serum starved cells were left untreated or treated with 10 ng/µl TGF-β before RNA extraction. mRNA expression levels were calculated relative to basal mRNA expression levels and expressed as -fold induction. (B) Total cell lysates were extracted from the cells treated and untreated with 10 ng/µl TGF-β over 0 hr, 18 hr and 24 hr. Western blot analysis was performed by using c-Myc antibodies. Protein loading was controlled using β-actin antibody.

The increased c-Myc expression after stimulation with TGF-β was caused by enhanced activity of the c-Myc promoter. Stimulation with TGF-β or transfection of a constitutive active TGF-β type-I receptor (TβRI) induced a 2 to 4-fold increase in c-Myc promoter activity, as revealed by reporter gene assays using a luciferase reporter construct that encompasses a 2.8 kb region of the human c-Myc promoter (Figure 7).

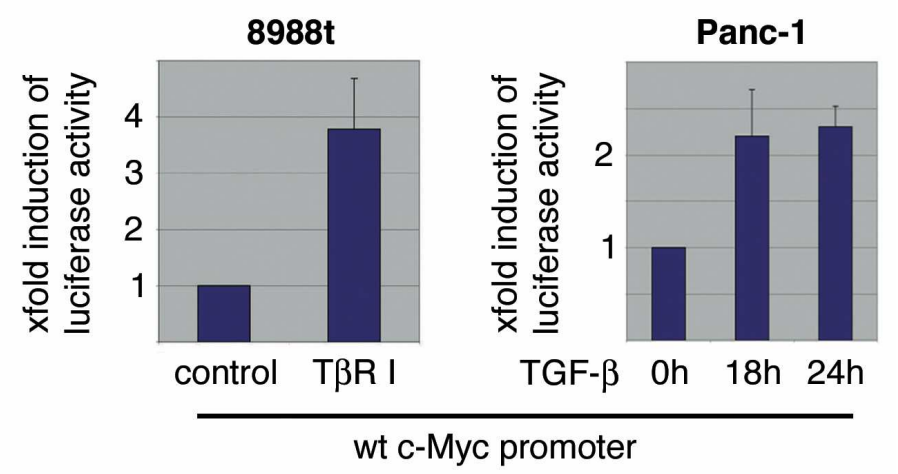

Figure 7) Transactivation of c-Myc promoter by TGF-β. Reporter gene assays illustrating the effect of TGF-β on human c-Myc promoter activity in 8988t and Panc-1 cells. Cells were transfected with a luciferase reporter gene construct containing the wild-type c-Myc promoter sequence along with Renilla luciferase plasmid and treated with 10 ng/µl TGF-β for 18 hr and 24 hr, in Panc-1 cells. 8988t cells were transfected with TβRI along with wild type c-Myc promoter. Firefly luciferase reporter gene activities were measured, normalized to TK-Renilla luciferase and expressed as mean -fold induction compared with untreated controls. Mean values were calculated from four independent experiments and are expressed as -fold induction.

To investigate the functional role of the TGF-β mediated c-Myc induction in cancer cells, we transientely knocked down c-Myc expression in Panc-1 cells by RNAi technology and examined the effect of TGF-β on cell growth in the absence of c-Myc. Dramatic reduction of cell proliferation was seen in proliferation assays after c-Myc knockdown, as compared to the cells transfected with control siRNA (Figure 8 – A and B). Interestingly, stimulation with TGF-β showed no effect on cell proliferation after knock-down of c-Myc in the tested cancer cells. Together, these results identified a novel mechanism of TGF-β in induction of cell proliferation by inducing c-Myc to promote cell growth in cancer cells.

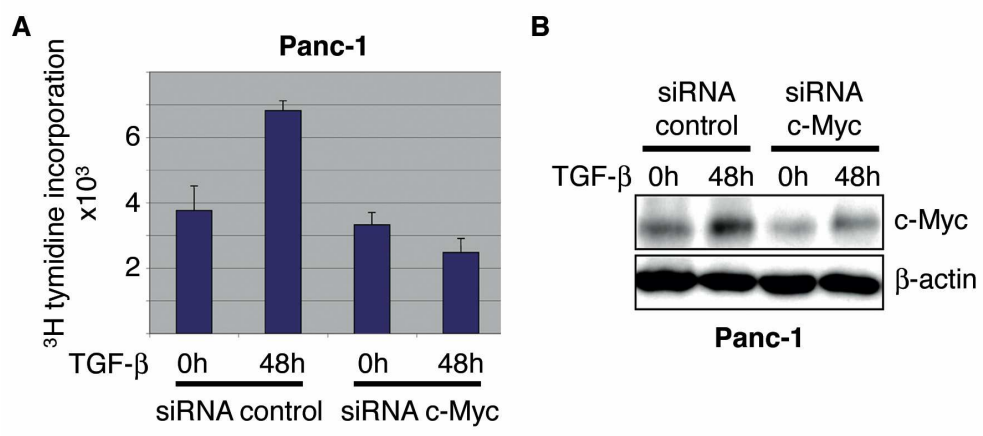

Figure 8) Silencing of c-Myc restores TGF-β antiproliferative behaviour. Reduction of c-Myc was confirmed on protein levels in Panc-1 cells after stimulation with TGF-β for 48 hr. (A) The relevance of c-Myc induction for TGF-β induced cell proliferation was assessed by [^{3}H] thymidine incorporation assays upon c-Myc silencing. Panc-1 cells were transfected with either control siRNA or siRNA against c-Myc in serum free medium. Starved cells were then incubated in serum free medium with or without 10 ng/µl TGF-β for 48 hr. Cell proliferation was assessed by incorporation of [^{3}H] thymidine in control and c-Myc knock down cells with and without TGF-β. Bars indicate mean values ± SD of three independent experiments. (B) Total cell lysate extraction of protein showed successful c-Myc knockdown as demonstrated by immunoblotting. Protein loading was controlled using anti-β-actin antibody. (Note that c-Myc depletion rendered cells refractory to TGF-β growth stimulation.)

4.3 NFAT TRANSCRIPTION FACTORS ARE ESSENTIAL FOR c-MYC INDUCTION BY TGF-BETA IN CANCER CELLS

To identify regulatory elements within the c-Myc promoter in response to stimulation with TGF-β, we used deletion constructs of the human c-Myc promoter shown in figure 9. Panc-1 cells were transfected with the indicated c-Myc promoter constructs (I-VI) along with TβRI. Interestingly, sequential deletion of the c-Myc promoter unveiled sustained TGF-β responsiveness up to the previously identified TIE (TGF-β inhibitory element, Figure 9). Deletion of the TIE

element (c-Myc del VI), however, impaired TGF-β inducibility of the c-Myc promoter in pancreatic cancer cells (Figure 9).

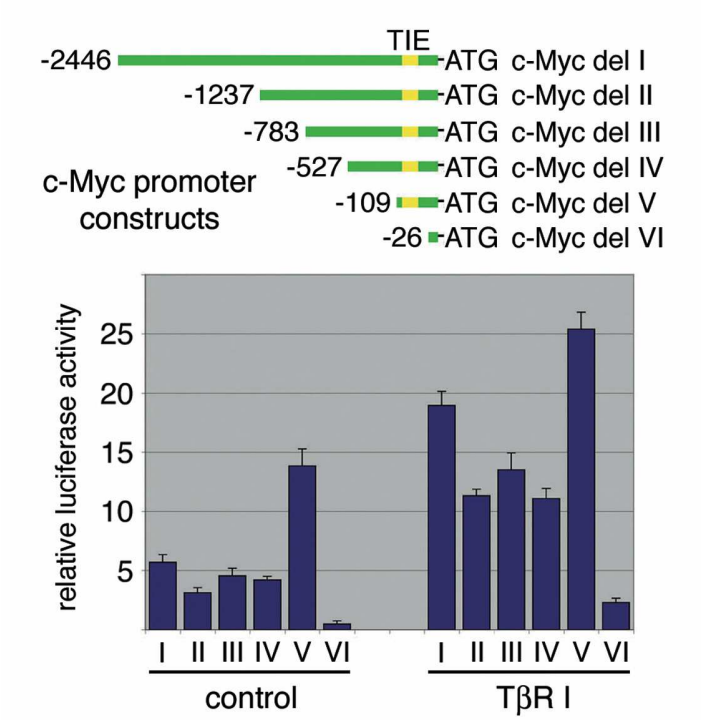

Figure 9) TGF-β increases the c-Myc expression by enhanced promoter activity. Panc-1 cells were co-transfected with either the full-length c-Myc promoter construct (c-Myc del I, -2446 to +334) or the indicated deletions constructs (c-Myc del II-VI) along with TβRI, or a control vector along with Renilla luciferase consturct. Firefly luciferase reporter gene activities were measured, normalized by TK Renilla luciferase and expressed as RLA (relative luciferase activities). Mean values were calculated from three independent experiments and are expressed as fold induction.

Similar to the results obtained with the 2.8 kb wild-type c-Myc promoter construct, TGF-β signalling either by transfection of TβRI plasmid or stimulation by TGF-β, increased the luciferase activity of the c-Myc promoter construct containing only the TIE element in growth promoted cell lines (Figure 10).

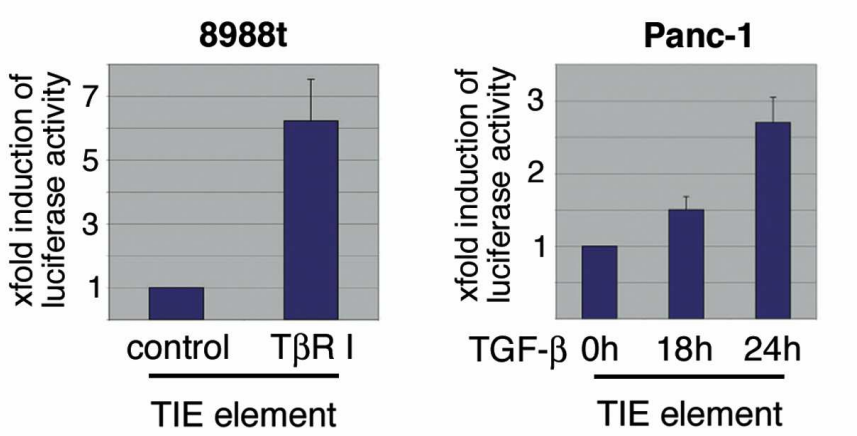

Figure 10) TGF-β increased c-myc expression is dependent on the TIE element within the c-myc promoter. 8988t and Panc-1 cells were transfected with the c-Myc/TIE reporter gene construct (-84 to -63) and TGF-β. signalling was initiated by either treatment with 10ng/µl TGF-β or co-transfection of TβRI along with Renilla luciferase plasmids. Firefly luciferase reporter gene activities were measured, normalized by TK Renilla luciferase and expressed as fold induction of luciferase activity. Reporter gene activities were expressed as mean fold induction compared to untreated control which was arbitrarily set to 1. Mean values were calculated from three independent experiments and are shown as mean ± standard deviation.

To elicit the functional relevance of the TTGG core sequence of the TIE element as well as the E2F and Smad binding sites within the TIE element of the c-Myc promoter in the context of the transactivation by TGF-β, we performed luciferase experiments using a c-Myc promoter construct containing a wild-type TIE element or a TIE element harboring several point mutants as demonstrated in figure 11. TGF-β inducibility of the c-Myc/TIE construct was accelerated upon mutational disruption of either the core sequence or the overlapping Smad/E2F interacting sequences, indicating that the repressor elements are potentially operative in proliferating cells (Figure 11, bars B-E). However, site directed mutagenesis of a neighboring NFAT binding motif (GGAAA, located between -75 and –71 relative to the P2 transcription initiation site) completely abolished TGF-β. responsiveness of the c-Myc/TIE construct (Figure 11, bar F).

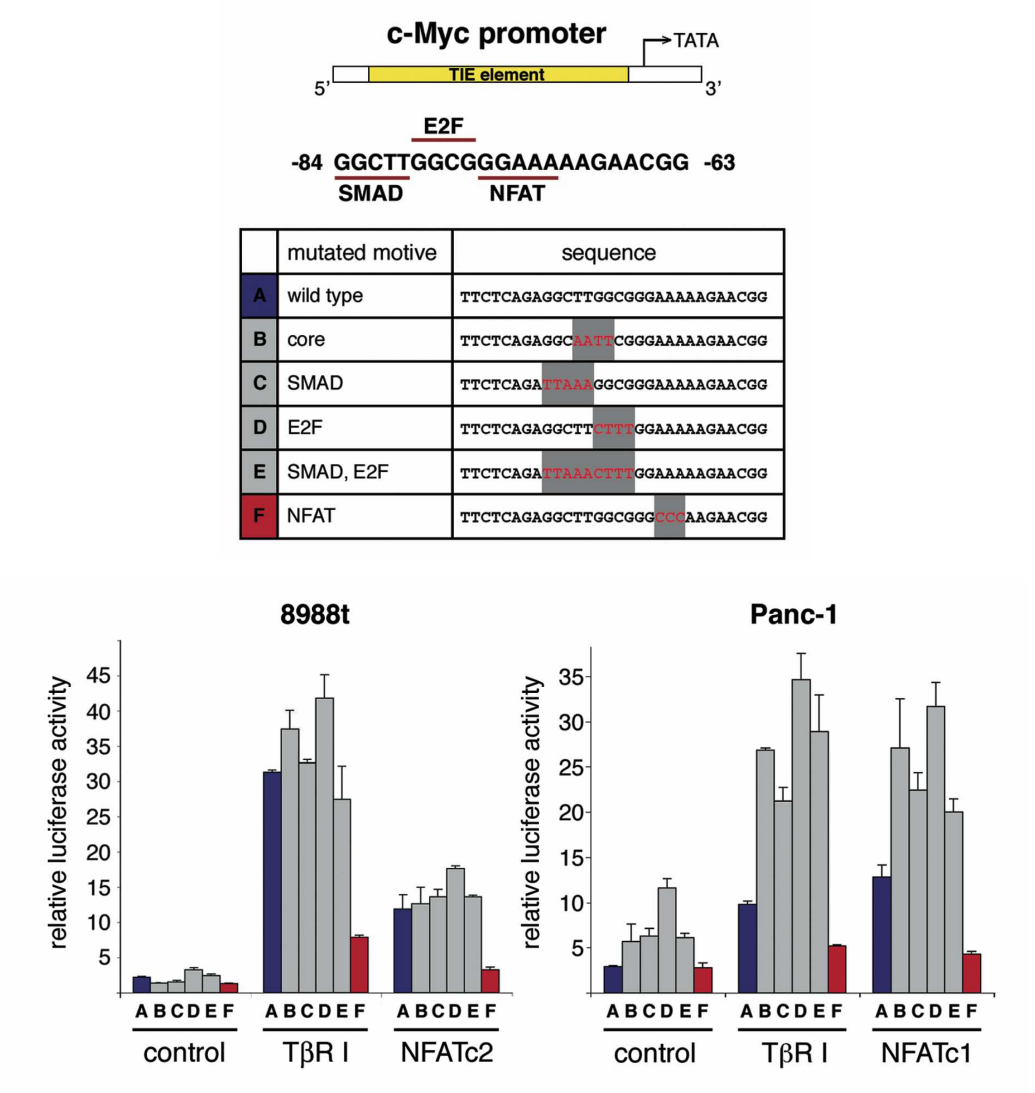

Figure 11) TGF-β increases c-Myc expression by enhanced promoter activity and is dependent on the NFAT consensus site within the TIE element. Schematic representation of the human c-*myc* TIE element including previously identified binding sites for SMADs and E2F4. The GGAAA NFAT-consensus sequence is also indicated. The table displayed the individual or combined mutations targeting these transcription factor binding sites as used for luciferase reporter gene assays (TIE mutants A-F). 8988t and Panc-1 cells were transfected with the indicated wild-type c-Myc/TIE construct (bar A in blue) or mutant c-Myc/TIE reporter constructs (bars B-F), along with TβRI or wild-type NFATc1/NFATc2 expression plasmids along with Renilla luciferase plasmid. Reporter gene activities were expressed as RLA (relative luciferase activities). Mean values were calculated from three independent experiments which were performed in triplicates. Bars indicate mean values ± standard deviation.

These findings suggested a role of NFAT factors in TGF-β induced c-Myc promoter activation in proliferating cells. To test the ability of NFAT to bind to the detected consensus sequence within the TIE-element, we performed DNA pulldown assays using a biotinylated double stranded oligonucleotides containing a wild-type NFAT binding sequence (c-Myc/TIEwt) and a mutated NFAT binding sequence (c-Myc/TIE-NFAT mut). Using the c-Myc/TIEwt oligonucleotides, we could detect NFAT bound to the DNA following stimulation with TGF-β in Panc-1 cells. But we could not detect NFAT binding to the c-Myc/TIE oligonucleotides following TGF- β stimulation when the NFAT consensus site was mutated (Figure 12). To confirm DNA binding of NFAT to its consensus sequence within the TIE-element after stimulation with TGF-β in an *in vivo* situation, we performed chromatin immunoprecipitation studies (ChIP) in Panc-1 cells using anti-NFATc1 antibodies. Rapid induction of NFAT binding to the full-length c-Myc promoter was observed after 3h of TGF-β stimulation (Figure 13). Additionally, a nearly 3-fold increase of NFAT binding to a 60 base pair fragment of the c-Myc promoter containing the TIE element (Figure 14) was detectable after stimulation with TGF-β β. Together, these results show a binding of NFAT to the TIE element within the c-Myc promoter following stimulation with TGF- β.

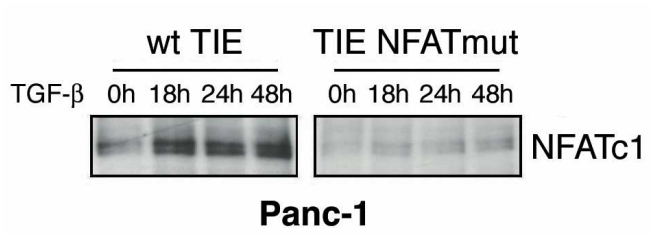

Figure 12) NFAT binds on c-Myc promoter within TIE element in the DNA pull down assay. DNA pulldown assays performed by using double-stranded oligonucleotides of wild-type and mutant TIE with disruption of the NFAT binding site. Panc-1 cells were serum starved and then treated with 10 ng/µl TGF-β for 0 hr, 18 hr, 24 hr and 48 hr. Nuclear extracts were prepared and incubated with either wt-TIE or TIE-NFATmut oligonucleotides. DNA–protein complexes were precipitated with

streptavidin–agarose beads and NFAT binding was analyzed by western blotting using NFATc1 antibodies.

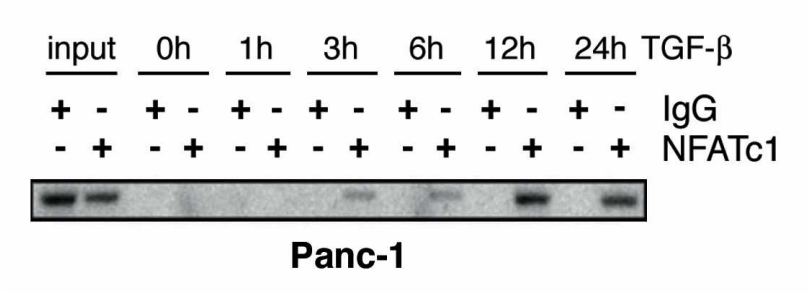

Figure 13) NFAT directly binds on c-Myc promoter. Chromatin immunoprecipitations were performed with specific NFATc1 antibodies in Panc-1 cells and *in vivo* binding to the c-Myc promoter was determined by semiquantitative PCR using primers specific for the full length c-Myc promoter region harboring the TIE.

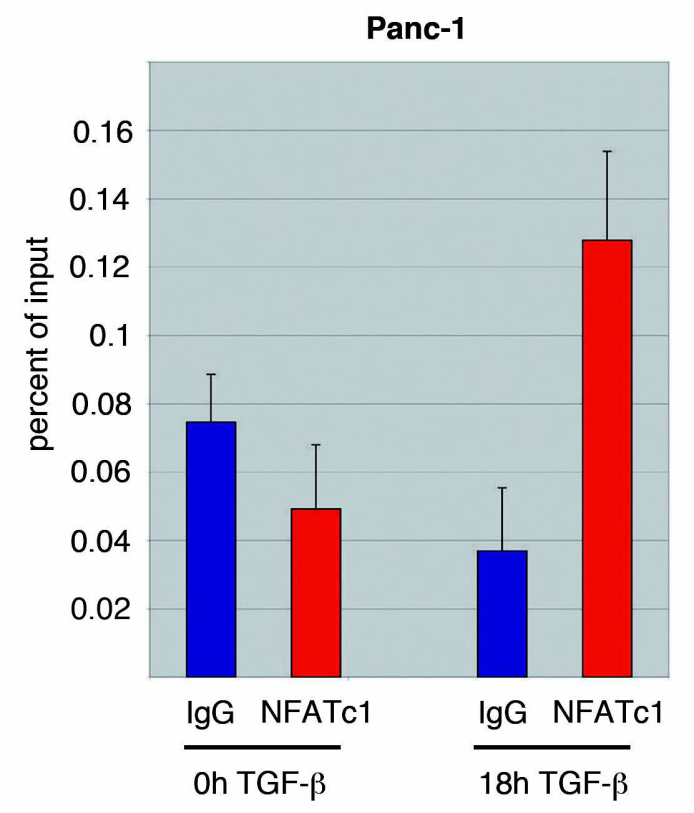

Figure 14) NFAT directly binds on c-Myc promoter. Chromatin immunoprecipitations were performed with specific NFATc1 antibodies in Panc-1 cells and *in vivo* binding to the c-Myc promoter was determined by real time PCR using primers specific for the 60 base pair within cMyc

promoter region harboring only the TIE. Mean values were calculated from three independent experiments which were performed in triplicates. Bars indicate mean values ± standard deviation.

4.4 TGF-BETA MODULATES NFAT ACTIVITY IN A SMAD-INDEPENDENT MANNER

Recent studies have demonstrated that NFAT proteins fulfill oncogenic functions and control critical mechanisms in proliferating cells during carcinogenesis (Buchholz et al., 2006; Medyouf & Ghysdael, 2008; Robbs et al., 2008). A basal expression of either NFATc1 or NFATc2 is detectable in both cell lines, however, none of the cell lines revealed co-expression of both transcriptional regulators. NFATc1 expression was detected in Panc-1 cells, whereas NFATc2 was found in 8988t (Figure 15). Following stimulation with TGF-β, the basal expression of NFATc1 and NFATc2 is strongly increased as shown in figure 15. Total cell lysates prepared from TGF-β treated cell lines and subjected to immunoblot analysis showed also an increased expression of Smad 3, which was more phosphorylated after TGF-β treatment showing an activation of the classical Smad signalling cascade in our cells. An expression of Smad4, which is increased after TGF-β stimulation, is only detectable in Panc-1 cells (Figure 15) because 8988t cells harboring a known Smad 4 deletion (Elsässer et al., 1992).

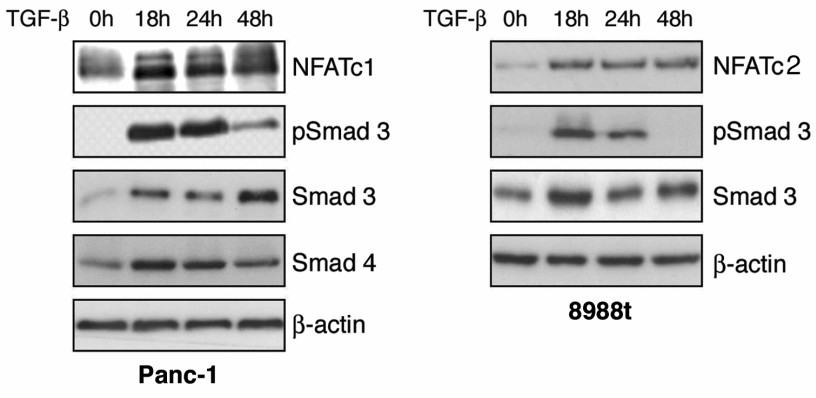

Figure 15) TGF-β induced expression of NFATc1/c2 in cancer cells. Total cell lysate extracts were prepared for western blot analysis to show time-dependent TGF-β mediated induction of NFAT factors in Panc-1 and 8988t cells. Cells were serum starved and then treated with 10 ng/µl TGF-β for the indicated time periods 0 hr, 18 hr, 24 hr, and 48 hr. Increased phosphorylation of Smad3 indicates successful treatment of TGF-β. Protein loading was controlled using β-actin antibodies.

More importantly, TGF-β treatment strongly induced the NFAT expression through promoter transactivation. Depending on the cell type and the duration of stimulation, TGF-β caused a 3 to 5 fold induction of the human NFATc1 or NFATc2 promoter as demonstrated in figure 21. These results suggest that TGF- β is able to increase NFAT expression by induction of the NFAT promoter in pancreatic cancer cells.

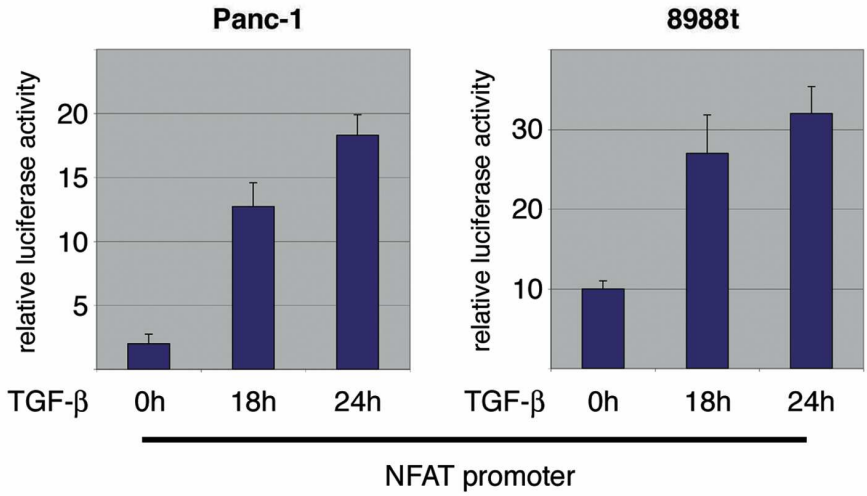

Figure 16) TGF-β induced the NFAT promoter transactivation. Reporter gene assays were performed in Panc-1 and 8988t cells following transfection of the human NFATc1 and NFATc2 promoters and treatment with 10 ng/µl TGF-β for 0 hr, 18 hr and 24 hr, respectively. Firefly luciferase reporter gene activities of the NFAT promoters were normalized to Renilla luciferase activity and expressed as RLA. Bars indicate mean values ± SD of three independent experiments performed in triplicates. Note that both NFAT promoters are induced by TGF-β treatment.

All studied cells, independent of the Smad4 expression status, respond to TGF-β treatment with increased activation of the NFAT promoter, which is followed by

enhanced expression of NFAT proteins. To test whether this enhancement of NFAT promoter activity is due to the expression of Smads, we performed luciferase reporter assays in both pancreatic cancer cell lines using NFATc1 and NFATc2 promoter construct together with the overexpression of Smad3 and Smad4. Neither individual transfection of Smad3 nor Smad4 enhanced the level of NFAT promoter activity in growth promoted cells (Figure 17), suggesting a Smad independent mechanism of NFAT induction by TGF-β.

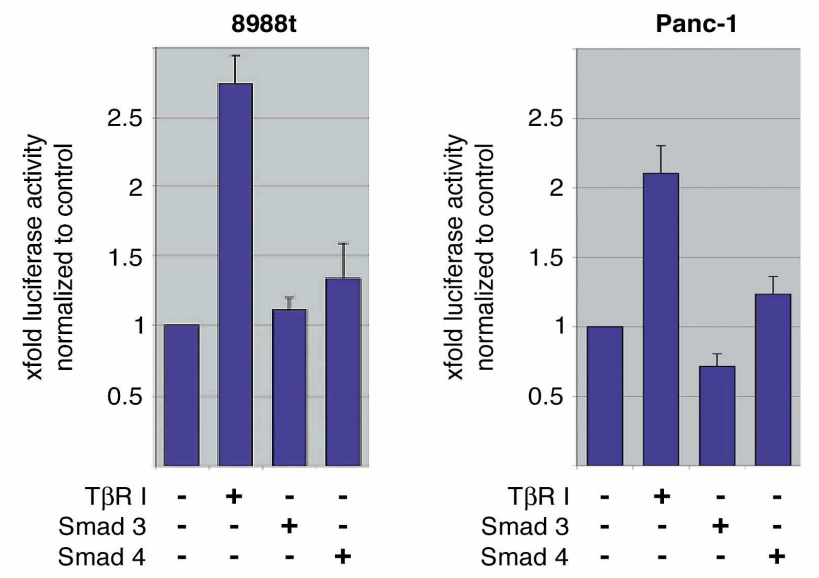

Figure 17) Smad3 and Smad4 can not transactivate the NFAT promoters. Reporter gene assay to define NFATc2 and NFATc1 promoter regulation by Smad3 and Smad4. 8988t and Panc-1 cells were transfected with the human NFATc2 and NFATc1 promoters respectively, along with either TβRI or Smads. Luciferase measured after 24 hr of transfection. Firefly luciferase reporter gene activities of the NFAT promoters were normalized to Renilla luciferase activity and expressed as RLA. Note that TGF-β induced NFAT promoters were not responsive to Smads. Fold induction compared to untreated control which was arbitrarily set to 1. Bars indicate mean values ± SD of three independent experiments performed in triplicates.

4.5 CALCINEURIN DEPENDENT TGF-BETA MEDIATED NFAT INDUCTION

As Smad overexpression is not able to increase NFAT promoter activity, we asked how TGF-β could induce NFAT expression in cancer cells. One potential signalling mechanism that is possibly critical for TGF-β mediated NFAT expression, is the activation of calcium-dependent molecules such as calcineurin. It has been shown before that calcineurin is required for the induction of several genes in response to TGF-β. Interestingly, an increased expression of calcineurin-B was observed in 8988t cells after TGF-β stimulation in a time dependent manner (Figure 18).

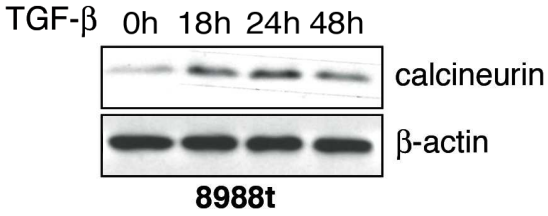

Figure 18) TGF-β mediated induction of calcineurin in pancreatic cancer cells. Induction of calcineurin-B was confirmed on protein levels in 8988t after stimulation with TGF-β 10 ng/µl for 0 hr, 18 hr, 24 hr and 48 hr. Total cell lysates were analyzed for calcineurin-B protein content using anti-calcineurin-B antibodies. Protein loading was controlled using β-actin antibodies.

NFAT proteins, which are basally phosphorylated and localized within the cytoplasm, are substrates of calcineurin (Medyouf et al., 2008). Consistent with increased phosphatase activity, the calcineurin substrates NFATc1 and NFATc2 became dephosphorylated and transported to the nucleus (Figure 19).

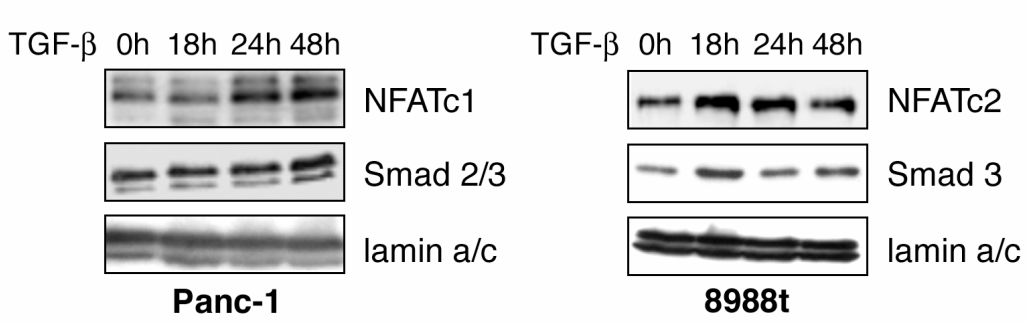

Figure 19) TGF-β induced calcineurin mediated translocation of NFATc1/c2 in the nucleus. 8988t and Panc-1 cells were used, after stimulation of TGF-β 10 ng/µl for 0 hr, 18 hr, 24 hr and 48 hr. Nuclear fractions were prepared and subjected to immunoblot analysis using anti-NFATc1, anti-NFATc2 and anti-Smad3 antibodies. Protein loading was controlled using lamina/c antibodies.

In contrast, application of cyclosporine A (CsA), an inhibitor of the phosphatase calcineurin, diminished NFAT induction by TGF-β on both promoter activity and mRNA level (Figure 20 and Figure 21), indicating that TGF-β utilize the canonical $Ca^{2+/}$calcineurin-signalling pathway to induce a positive NFAT feedback loop in proliferating cells. Inhibition of calcineurin prior to the addition of TGF-β revealed that calcineurin is required for TGF-β mediated NFAT induction in cancer cell. Altogether these results showed that the NFAT promoter activation by TGF-β occured in a Smad independent manner but requires an active calcineurin phosphatase pathway.

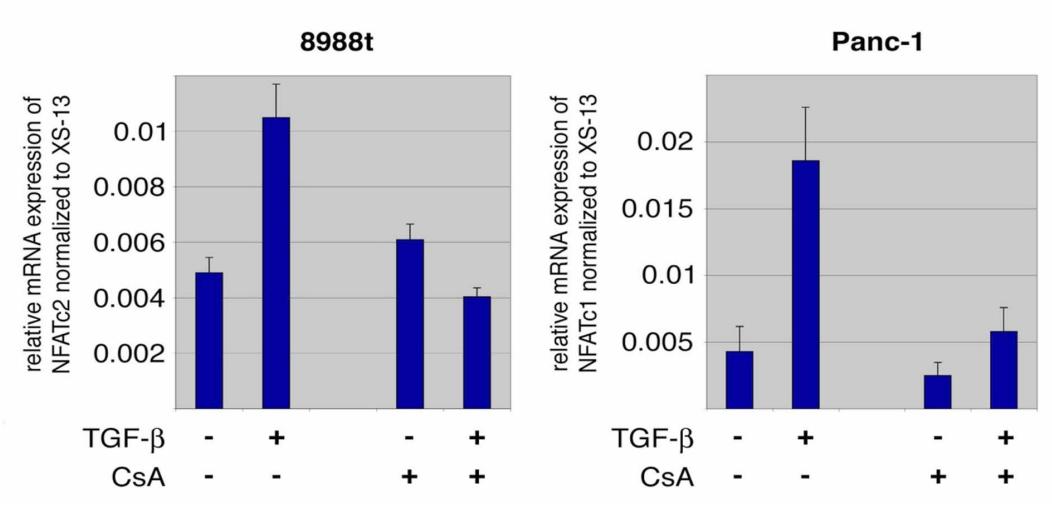

Figure 20) TGF-β induced NFATc1/c2 mRNA expression in pancreatic cancer cells by calcium influx in the cell. TGF-βmediated induction of NFAT mRNA expression was analyzed by Real Time-PCR in Panc-1 and 8988t cells. Initially, cells were treated with CsA in serum free medium. Then, serum starved cells containing CsA were left untreated or treated with 10 ng/µl TGF-β. RNA extraction was prepared 24 hr after TGF-β stimulation. mRNA expression levels were calculated relative to basal mRNA expression, which were arbitrarily set to 1 for each experiment, and expressed as fold-induction. Bars indicate mean values ± SD of three independent experiments performed in triplicates.

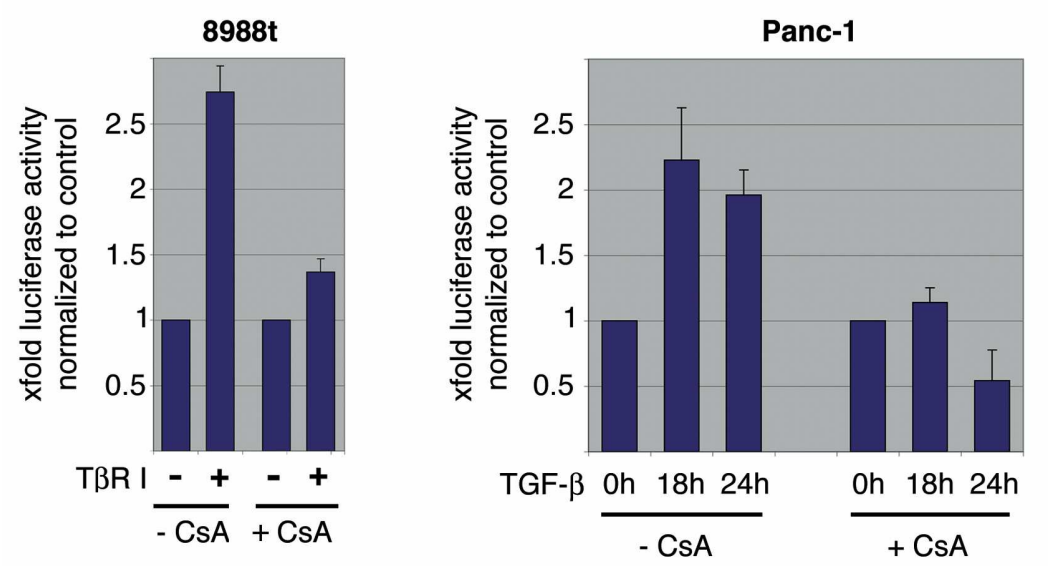

Figure 21) Cyclosporin rescued the TGF-β mediated transactivation of NFATc1/c2 promoter in cancer cells. Reporter gene assays to define NFATc1 and NFATc2 promoter regulation by

cyclosporine A (CsA) treatment. Cells were transfected with the human NFATc2 promoter along with TβRI and incubated in the presence or absence of CsA to block endogenous calcineurin activity. Note that TGF-β induced NFATc2 and NFATc1 promoter activation was antagonized by pharmacological inhibition of calcineurin. Bars indicate mean values ± SD of three independent experiments performed in triplicates.

4.6 REQUIREMENT OF NFAT FOR TGF-BETA MEDIATED c-MYC INDUCTION IN CANCER

To identify the importance of NFAT for TGF-β mediated c-Myc induction on promoter level, we performed reporter assays in the presence and absence of CsA. Therefore, cells were transfected with a c-Myc/TIEwt promoter construct and treated with the calcineurin inhibitor CsA. Surprisingly, TGF-β mediated activation of c-Myc/TIEwt promoter construct was abolished after treatment with CsA (Figure 22).

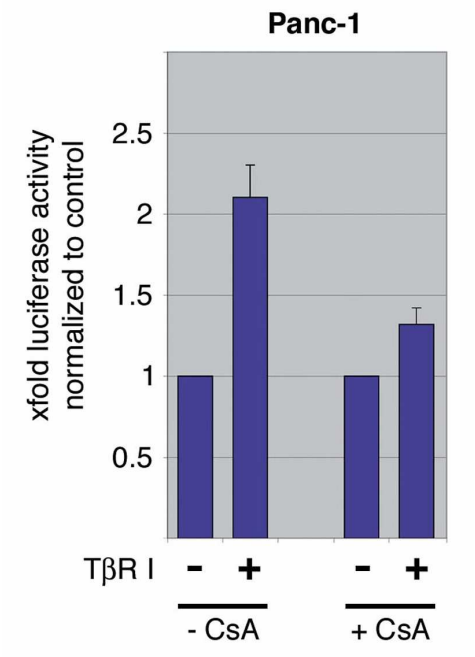

Figure 22) Cyclosporin rescued the transactivation of c-Myc promoter by TGF-β. Reporter gene assays to define c-Myc/TIEwt promoter regulation by cyclosporine A (CsA) treatment. Cells were transfected with the human c-Myc promoter along with either TβRI and incubated in the presence

or absence of CsA. Luciferase was measured after 24 hr of transfection. Firefly luciferase reporter gene activities of the c-Myc/TIE promoter were normalized to Renilla. Data are represented of triplicate experiments and are displayed as bars ±SD.

To investigate the role of NFAT in the context of TGF-β mediated c-Myc induction in more detail, we performed NFAT knock down experiments in the presence of TGF- β. Twenty-four hours after transfection with control siRNA or NFAT siRNA, cells were incubated with TGF-β for up to 48 hours. As shown in figure 23A, knock-down of NFAT prevents the induction of c-Myc mRNA by TGF-β (Figure 23A). This prevention of c-Myc induction was also reflected on protein level, as shown in figure 23B. In cells transfected with control siRNA, increased NFAT protein is detectable 18 hr after TGF-β treatment while c-Myc enhancement starts after 24 hr of TGF-β treatment. In case of NFAT knock-down neither an increase of NFAT nor of c-Myc was detectable under treatment with TGF- β (Figure 23B). These results support the assumption that c-Myc is a transcriptional target of NFAT in cancer cells and suggest a role for NFAT in TGF-β mediated proliferation.

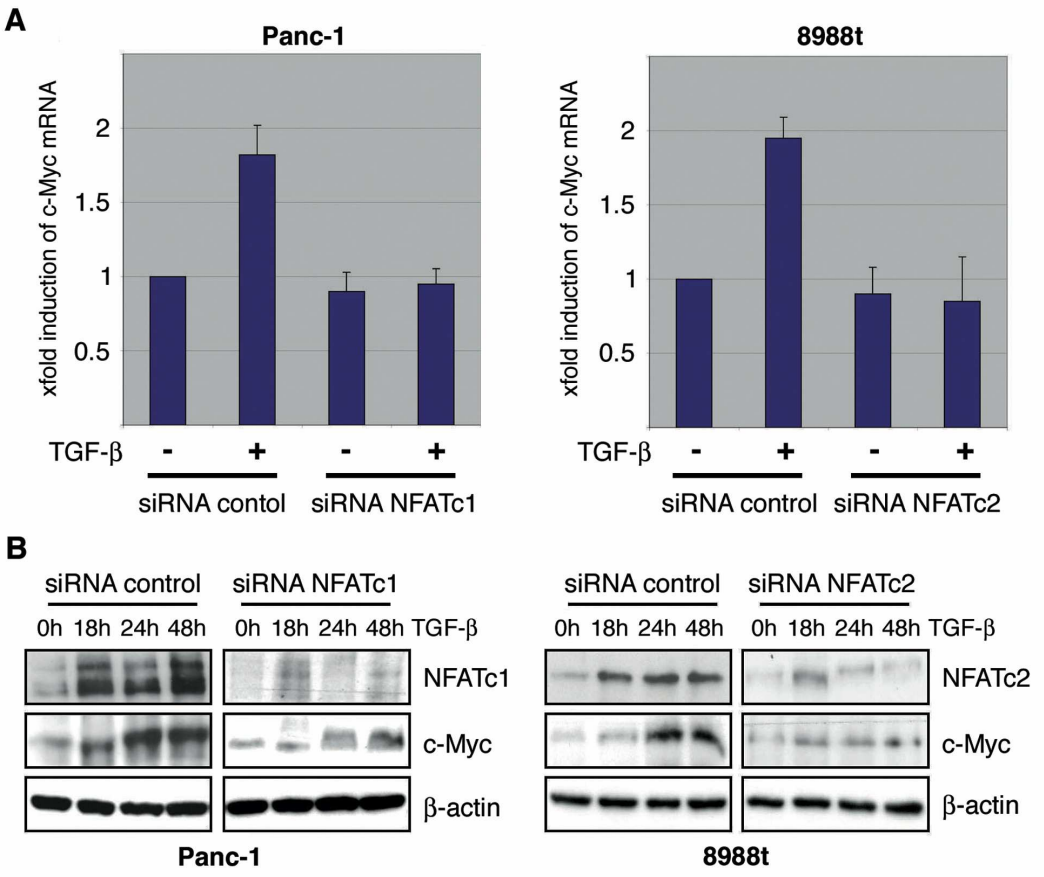

Figure 23) NFAT expression required for c-Myc induction by TGF-β. (A) Cancer cells were transfected with control siRNA or siRNA against NFAT proteins 24 hr post transfection, cells were serum starved and then treated with either serum free medium or medium containing 10 ng/µl TGF-β. cDNA was prepared and subjected to qRT–PCR to analyze the effect of NFAT knockdown on c-Myc mRNA expression in Panc-1 (left) and 8988t (right) cells. Values were calculated relative to basal mRNA expression levels in control siRNA transfected cells, which were arbitrarily set to 1 for each experiment. Displayed are mean values from three independent experiments ± standard deviations. (B) TGF-β treated Panc-1 and 8988t cells transfected with either non-specific control siRNA or NFATc1-siRNA and NFATc2-siRNA respectively. Total cell lysate proteins were extracted and subjected to immunoblotting with antibodies specific for NFATc1, NFATc2 or c-Myc. Protein loading was controlled using β-actin antibodies.

To elucidate the role of the TGF-β/Smad signalling pathway, which is activated in response to TGF-β treatment in our pancreatic cancer cells, in the context of c-Myc induction we performed luciferase reporter assays and western blot analysis

after genetic knock-down of Smad3. We can show that knock-down of Smad3 did neither prevent TGF-β induced expression of NFATc2 and NFATc1 (Figure 24), nor the subsequent induction of c-Myc. The knock-down of Smad also failed to prevent activation of the c-Myc promoter following activation of TGF-β signalling (Figure 25). Moreover, TGF-β mediated induction of the c-Myc promoter is even more enhanced after knock-down of Smad3 (Figure 25). These studies show that TGF-β induced and NFAT mediated induction of c-Myc is completely independent of Smad signalling.

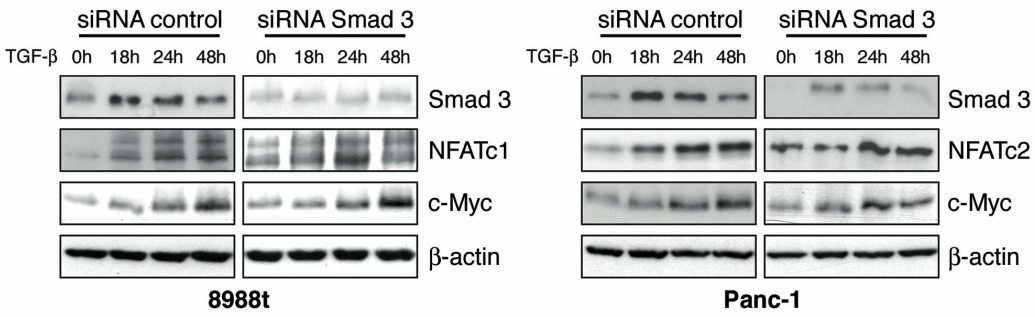

Figure 24) Loss of Smad3 did not affect NFAT and c-Myc expression. Panc-1 and 8988t cells were transfected with siRNA against Smad3 or control siRNA, serum starved cells treated in serum free medium alone or medium containing 10 ng/µl TGF-β for 0 hr, 18 hr, 24 hr or 48 hr. Total protein lysates extracted and immunoblot analysis were performed to determine successful depletion of Smad3 and its impact on the expression levels of NFAT and c-Myc in cancer cells. Protein loading was controlled using β-actin antibodies. Note that c-Myc expression was not affected following depletion of Smad3.

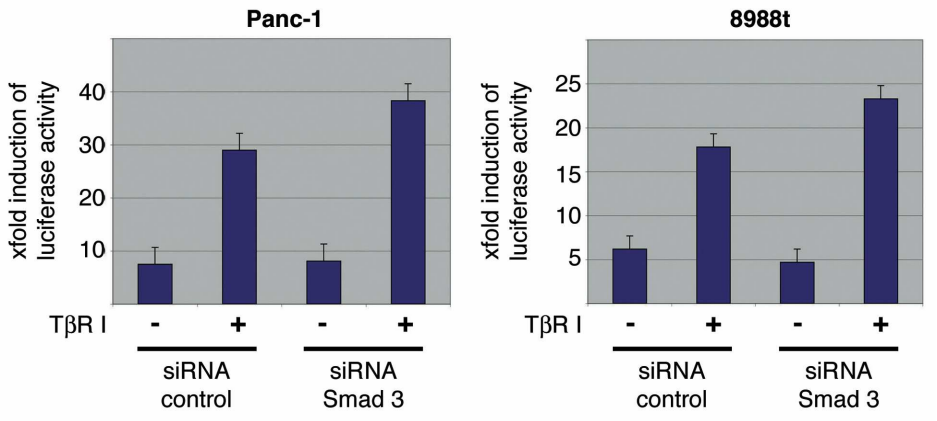

Figure 25) Loss of Smad3 did not affect transactivation of c-Myc promoter. Reporter gene assays were performed in Panc-1 and 8988t cells following transfection of unspecific siRNA or siRNA against Smad3 along with c-Myc/TIE reporter construct along with TβRI plasmid. c-Myc promoter activities were expressed as RLA. Firefly luciferase reporter gene activities of the c-Myc/TIE promoter were normalized to Renilla. Data are represented of triplicate experiments and are displayed as bars ±SD. (Note that deletion of Smad3 did not affect TGF-β inducibility of the c-Myc promoter.)

4.7 NFAT ANTAGONIZES THE SMAD3 REPRESSOR COMPLEX TO ACTIVATE THE c-MYC PROMOTER

Recent studies in growth-inhibited cells convincingly demonstrated that Smad3 binding to the TIE element is a prerequisite for c-Myc repression and results in growth inhibition (Chen et al., 2002). To learn more about the interplay between NFAT and Smad3 on the level of the c-Myc promoter, we performed reporter gene assays using a wild type and a mutated TIE-element. As demonstrated in figure 26, Smad3 is active on the c-Myc/TIE element in cancer cells and represses the activity of a c-Myc promoter construct. Stepwise overexpression of NFATc1 or NFATc2 in increasing concentrations however is able to antagonize the effect of Smad3 mediated repression on c-Myc promoter. In contrast, NFAT failed to antagonize the Smad3 mediated repression of the c-Myc promoter in both the pancreatic

cancer cell lines by using a c-Myc promoter construct which harbors a mutated NFAT consensus sequence (Figure 26).

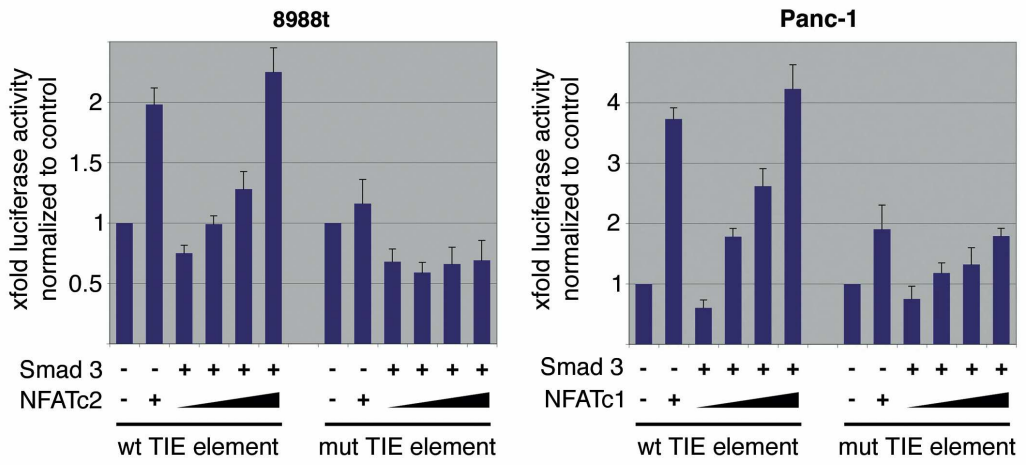

Figure 26) Competition between Smad3 and NFAT for the c-Myc promoter. Reporter gene assay to analyze c-Myc/TIE promoter induction depending on the integrity of the NFAT binding site and the amount of NFAT and Smad3. 8988t and Panc-1 cells were transfected with c- Myc/TIE wild-type or c-Myc/TIE-mutant that lacks the NFAT binding site and expression plasmid of Smad3 wild type along with increasing amounts of NFATc2. c-Myc promoter activities were expressed as mean fold induction. Firefly luciferase reporter gene activities of the c-Myc/TIE promoter or c-Myc/TIE mutant were normalized to Renilla. Mean value were calculated from three independent experiments and are shown as mean ± SD

According to the antagonistic effect of NFAT to the Smad mediated repression of the c-Myc promoter, we hypothesized that induction of NFAT by activation of the TGF-β pathway interfered with Smad3 promoter binding and displaced this transcription factor from the TIE element within the c-Myc promoter. To confirm our hypothesis, we performed DNA pulldown experiments by using a wild-type c-Myc/TIEwt biotinylated double stranded oligonucleotides probe and chromatin immunoprecipetation (CHiP) experiments with the precipitation of NFATc2 or Smad3 on c-Myc promoter. As known from former experiments, both NFAT and Smad3 were induced upon TGF-β stimulation in a time dependent manner (Figure

15). As shown in figure 27, *in vitro* (A) and *in vivo* (B) binding of Smad3 to the c-Myc/TIE element was found in all cancer cells when they were cultured in the absence of TGF-β. However, Smad3 binding to the c-Myc promoter was replaced by NFAT from the c-Myc promoter in a time dependent manner following TGF-β treatment. Here we have shown that increasing amounts of NFAT overrides Smad3 mediated repression of the c-Myc promoter. Together, these experiments show an antagonistic interplay between NFAT factors and Smad3 in the TGF-β mediated induction of c-Myc in pancreatic cancer cells.

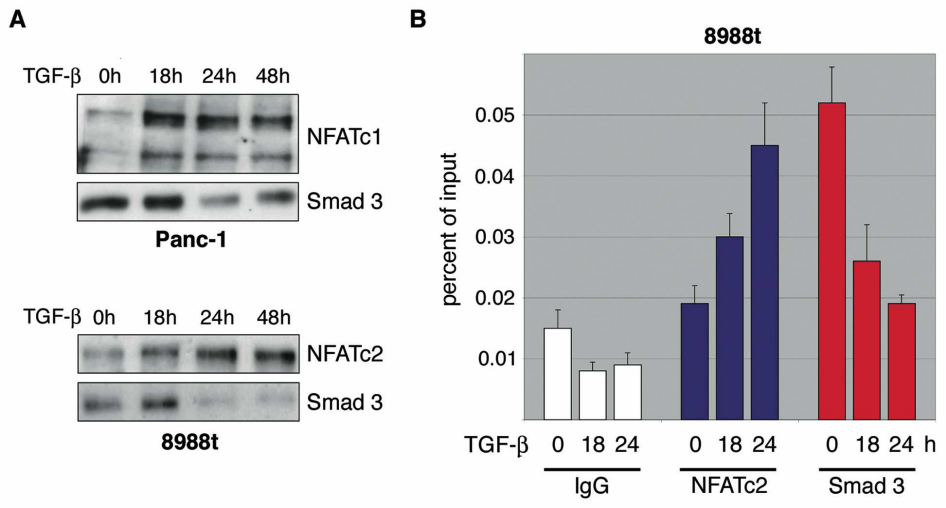

Figure 27) NFAT displaces Smad3 from the c-Myc/TIE upon TGF-β. (A) DNA pulldown experiment demonstrating inverse binding of NFAT factors and Smad3 on the c-Myc/TIE upon TGF-β. Nuclear extracts from Panc-1 (upper panel) and 8988t (lower panel) cells were prepared and incubated with the wild-type c-Myc/TIE oligonucleotide sequence. DNA–protein complexes were precipitated with streptavidin–agarose beads, and NFAT/Smad3 binding was analyzed by western blotting using anti- NFATc1 and anti-Smad3 antibodies, respectively. Note the inverse binding of NFAT factors and Smad3 on the c-Myc/TIE sequence upon treatment with TGF-β. (B) Chromatin immunoprecipitations were performed in 8988t cells following 10ng/ul TGF-β treatment over 18 hr and 24 hr by using specific NFATc2 and Smad3 antibodies. I*n vivo* binding of both transcription factrs to the c-Myc promoter was determined by quantitative PCR using primers specific for the c-*myc* promoter region harboring the c-Myc/TIE. ChIP assay demonstrates inverse binding of Smad3 and NFAT to the c-Myc/TIE upon TGF-β stimulation.

4.8 NFAT MEDIATES THE TGF-BETA SWITCH FROM A GROWTH SUPPRESSOR TO A PROMOTER OF CELL PROLIFERATION

Finally, we investigated the functional relevance of NFAT in TGF-β promoted cell cycle progression and tumor cell growth. Similar to the effects of c-Myc knock-down (demonstrated in Figure 8), depletion of NFAT fully prevented growth stimulation by TGF-β in both pancreatic cancer cell lines (Figure 28 –A and B). The importance of NFAT in response to TGF-β treatment is demonstrated by proliferation assays after knock-down of NFAT. Firstly, basal cell proliferation was decreased after NFAT knock-down. But more surprisingly, the TGF-β mediated induction of cell proliferation was completely lost after knock-down of NFATc1 or c2 in both pancreatic cancer cell lines (Figure 28 A).

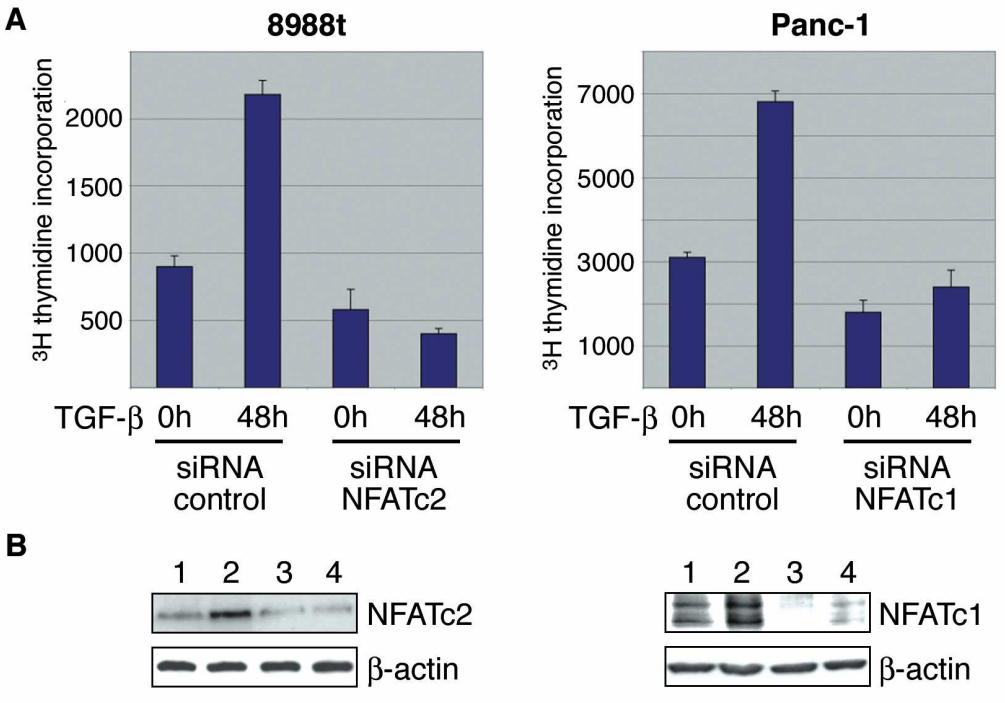

Figure 28) NFAT proteins mediate the TGF-β switch from growth suppressor to a promoter of cell proliferation. (A) The relevance of NFAT expression for TGF-β induced cell proliferation was

assessed by [^{3}H] thymidine incorporation assay upon NFAT silencing. 8988t and Panc-1 cells were transfected with either control siRNA or siRNA against NFATc2 and NFATc1 respectively. Cells were starved and incubated in serum free medium with or without 10 ng/µl TGF-β for 48 hr. Bars indicate mean values ± SD of three independent experiments. Note that NFAT depletion rendered cells refractory to growth stimulation and partially restored TGF-β growth suppressor activities in 8988t and Panc-1 cells. (B) Total cell lysate protein was extrated from the 8988t and Panc-1 cells, using either control siRNA or siRNA against NFATc2 or NFATc1 respectively and then treated with TGF-β 10 ng/µl. Successful NFAT knock-down was demonstrated by immunoblotting using specific antibodies against NFATc1 and NFATc2 (bottom panel: control siRNA (lane1), control siRNA + TGF-β (lane2), siRNA NFAT (lane3) and siRNA NFAT + TGF- β (lane4). Protein loading was controlled by using β-actin antibodies.

Moreover, NFAT silencing partially restored the growth inhibitory response of cancer cells to TGF-β treatment (Figure 28), as evidenced by down-regulation of the cell cycle regulatory genes, especially cyclin D3 and CdK6, and an increased halt of cancer cells in G1 upon TGF-β treatment (Figure 29 and Figure 30). Taken together, these findings establish NFAT proteins as central mediators in TGF-β induced tumor cell growth and strongly support a key role of these transcription factors in the molecular switch controlling TGF-β growth response.

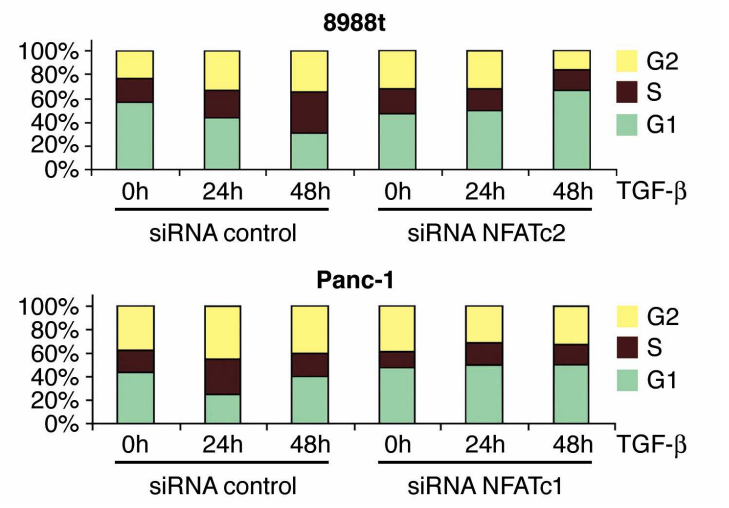

Figure 29) Silencing of NFAT restores TGF-β induced G1/S phase transition. Flow cytometry analysis to study the relevance of NFAT factors in TGF-β induced cell cycle progression of cancer

cells. NFAT knockdown cells (Panc-1 and 8988t) were treated with TGF-β 10ng/ul for 24 hr or 48 hr, respectively, and analyzed by propidium iodide staining and flow cytometry. Cell cycle stages are illustrated in different colors: G2 (yellow), S (brown), and G1 (green). Loss of NFAT expression restored cell cycle inhibition by TGF-β, as evidenced by increased cells in the G1 phase. Bars indicate mean values ± SD of three independent experiments

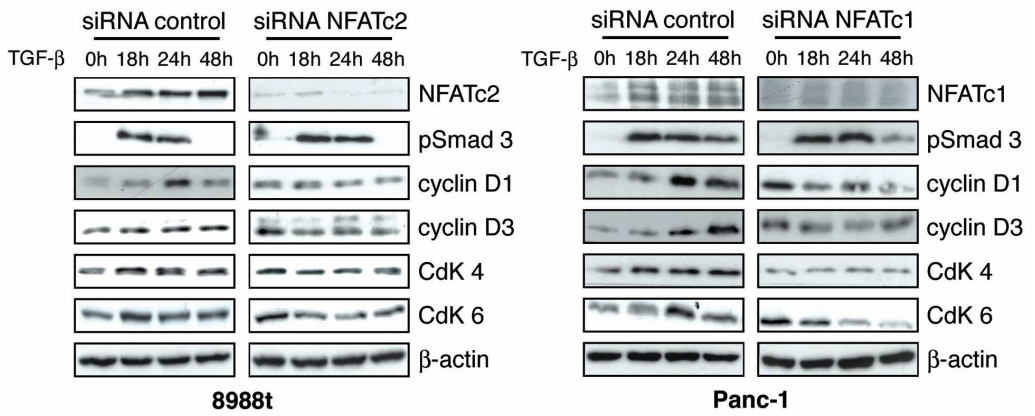

Figure 30) knock down of NFAT prevents induction of Cyclins by TGF-β. Total cell lystes were extracted for western blot analysis demonstrating TGF-β regulated cell cycle genes depending on the presence or absence of NFAT expression. Cells were transfected with siRNA against NFATc2 or unspecific control siRNA, serum starved and subsequently treated with 10 ng/µl TGF-β for 0 hr, 18 hr, 24 hr, or 48 hr. Total cell lysates were then analyzed for expression of NFAT, D-type cyclins and their partnering kinases (CdKs). Protein loading was controlled by using β-actin antibodies.

5 DISCUSSION

Pancreatic cancer is the 4th leading cause of cancer-related death in western countries. The number of diagnoses per year equals the number of deaths per year, making it the deadliest of all malignancies. This is due in part to the low rate of respectability at presentation and very aggressive tumor behavior with an overall dismal prognosis. Although surgical resection is the only treatment method which offers the possibility of a cure, there are only a handful of long-term survivors. The poor survival of this malignancy has stimulated a wealth of research efforts into understanding the molecular mechanisms responsible for this disease. Results of these efforts have led to the discovery of the role of many molecular signalling systems involved in pancreatic carcinogenesis (Friess et al., 1999).

The transforming growth factor-β (TGF-β) pathway is one of the signalling system that has been identified as a major contributor (Ghaneh et al., 2009; Friess et al., 1993). TGF-β is an important cytokine that is involved in an extraordinary range of biological processes. At its discovery in 1983, it was given its malevolent moniker as a 'transforming' factor because of its ability to transform rat fibroblasts (Anzano et al., 1983; Chin et al., 2004). However, it is now known that TGF-β is a very helpful cytokine and actually is one of the most potent inhibitors of cellular proliferation in normal cells. Although limiting cellular proliferation is one of the primary functions of the TGF-β-signalling pathway in normal epithelial cells, numerous other cellular responsibilities have been identified, including but not limited to embryogenesis, differentiation, apoptosis, angiogenesis, immunosuppression and wound healing (Massague et al., 2000).

The growth inhibiting functions of TGF-β are mainly based on its potential to arrest cells at G1 phase or to induce programmed cell death (Hannon et al., 1994).

Although TGF-β can stop cell cycle transition at any point, these responses are only effective at inhibiting cell cycle progression during G1. Specifically, TGF-β is able to prevent this progression by inducing the expression of the cyclin kinase inhibitors such as p15, p21, and p27 among others (Polyak et al., 1994; Datto et al., 1995; Hannon et al., 1994). These inhibitors block cyclins and CdKs from phosphorylating Rb and thereby inhibiting G1/S progression. In addition to controlling cell cycle progression through the induction of cyclin kinase inhibitors, TGF-β also directly suppresses mitogenic c-myc expression (Pietenpol et al., 1990a; Pietenpol et al., 1990b). c-Myc is a ubiquitous promoter of cell growth and proliferation and its transcriptional downregulation is a rapid and general effect observed in most cells with an antiproliferative response to TGF-β. c-Myc levels must be low in order for powerful cyclin kinase inhibitors to become activated by TGF-β, since normally c-myc binds to the promoters of the cyclin kinase inhibitors (P15, P21 etc.) thus preventing their activation (Seoane et al., 2004). In most epithelial cells, including pancreatic ductal cells, TGF-β leads to the repression of c-Myc levels and the expression of cyclin kinase inhibitors, which in combination, achieve substantial inhibition of the G1 to S phase progression (Dang et al., 1999).

During carcinogenesis, most epithelial tumors cells change their responsiveness to TGF-β, escape from growth inhibition and instead respond with a more aggressive phenotype (Bachman et al., 2005; Tang et al., 2003). Much of our current understanding of this duplicitous role of TGF-β has been carried out in models of skin carcinogenesis where it has been best established (Li et al., 2005; Wang et al., 2001; Li et al., 2006). Although the exact detailed mechanisms of this functional switch and its regulation remain to be elucidated, overexpression of the TGF-β is commonly implicated. Most cancer cells secrete larger amounts of TGF-β than their normal cell counterparts, and this overexpression is strongest in the most advanced stages of pancreatic and other malignancies (Derynck et al., 1985; Glynne-Jones et al., 1994).

Overexpression of TGF-β induces epithelial to mesenchymal transition (EMT) of normal and transformed epithelial cells and thus an enhanced proliferation and the migratory ability (Ellenrieder et al., 2001). This increased migration by epithelial cells that have undergone EMT is mediated by repression of cell–cell adhesion proteins, such as E-cadherin, and the induction of invasion-associated integrins and integrin-binding proteins. On the morphologic level, EMT results in the formation of highly aggressive, totally fibroblastic spindle carcinoma that have lost all the molecular markers of epithelial cells (Elliott et al., 2005). Changes in motility elicited by cytoskeletal re-organization, and enhanced secretion of matrix-remodeling enzymes are classically considered the main driving forces in the contribution of reversible TGF-β-driven EMT to invasion and metastasis (Derynck and Zhang et al., 2003; Ellenrieder et al., 2008, Yu et al, 2002).

In pancreatic cancer, TGF-β mediated EMT is also closely associated with a strong promotion of cancer cell growth, although the molecular mechanisms underlying this phenomena are basically unknown. With the goal to dissect the mechanisms behind TGF-β's ability to regulate these two opposing effects on pancreatic cancer cell growth, we conducted the presented study and uncovered a novel transcriptional pathway that mediates the TGF-β-growth switch from a suppressor to a promoter of cell proliferation. We initially demonstrated the growth stimulatory potential of TGF-β, which was apparent after 24hr and reached significance after 48hr post treatment. Growth stimulation was based on accelerated G1/S phase transition of the cell cycle and reflected by increased expression of D-type cyclins (cyclin D1 and cyclin D3) and their corresponding kinase partners CDK4 and CDK6.

Most importantly, growth stimulation by the growth factor was dependent on a sustained induction of the c-Myc oncogene. In fact, we provide strong evidence that TGF-β strongly induces transcription from the c-Myc promoter and this

function is essential for subsequent G1 cell cycle transition, as evidenced by knockdown experiments. Detailed analysis of c-myc promoter regulation further showed that TGF-β targets a short promoter element that was previously described as the core element in promoter silencing by TGF-β. This core element, called TGF-β inhibitory element (TIE) is located between –84 and -63 relative to the P2 transcription initiation site (Chen et al., 2001) is essential for promoter repression. Extensive promoter analysis studies have demonstrated that in growth inhibited epithelial cells, TGF-β represses the c-Myc/TIE element through a TTGG-core sequence, which combines an E2F-binding site with a Smad (GCTT) interacting motif. Site directed mutagenesis revelead that c-Myc repression is mediated by Smad3 bound repressor complexes. Smad3 interacts with p130 and E2F4 co-repressor proteins, binds to the TIE element and silence the promoter activity in order to block cell cycle transition (Chen et al., 2001).

Our group have recently uncovered an alternative route of c-Myc suppression that is mediated by Smad3 interaction with the Sp1/KLF like transcription factor KLF11. KLF11 itself is induced by TGF-β and then binds to nuclear Smad3 (Buck et al., 2006). Both factor form complexes in response to TGF-β and repress the c-Myc promoter through interaction with the TIE element, again underscoring the outmost importance of this promoter regulatory region. We now provide evidence that TGF-β aims at the identical c-Myc/TIE promoter sequence to promote pancreatic cancer cell growth, in this case however, to stimulate rather than suppress the promoter. On the molecular level, TGF-β induced the c-Myc promoter through the NFAT signalling and transcription pathway.

NFAT proteins a family of transcription factors, comprises five members four out of them are Ca^{2+}/calcineurin regulated proteins and one of them is NFAT5 (TonEBP: tonicity element binding protein), which is regulated by hyperosmotic stress and integrins (Rao et al., 1997; Serfling et al., 2007). Nuclear factor of

activated T cells (NFAT) has originally been identified as an inducible nuclear factor which binds to, and upregulates transcription from, the interleukin-2 (IL-2) gene promoter in T cells following stimulation of the T cell antigen receptor. Following their initial discovery, a multitude of studies quickly established that NFAT proteins are expressed not only in T cells, but many different cells of the immune system, and that signalling through NFAT proteins is crucial for the development and function of the immune system (Feske et al., 2003; Macian et al., 2005).

In resting cells, NFAT is located in the cytoplasm as a hyperphosphorylated, inactive form. Under these conditions, NFAT phosphorylation is insured by the combined action of several maintenance kinases, including CK1 and DYRK2 that target specific serine residues in the NFAT conserved regulatory domain (Rao et al., 1997). Signalling through calcium/calcineurin results in calcineurin-dependent dephosphorylation of NFAT proteins, allowing their subsequent nuclear translocation, where they bind to their specific DNA binding 9 base pair element having consensus nucleotide sequence (A/T)GGAAA(A/N)(A/T/C)N to regulate the transcription (Rao et al., 1997; Medyouf & Ghysdael 2008). Since NFAT proteins bind their DNA target sequences only with relatively weak affinity, they regulate many, and probably all, of their target gene promoters in cooperation with partnering transcription factors.

NFAT transcription complexes thus can be seen as integrators of signals from different pathways, where one signal must Ca2+/calcineurin, while the other signal(s) may be from divers other pathways. This also explains, in part, the broad diversity of cellular responses that Ca2+ signalling triggers depending on the cell type or the state of cellular differentiation and/or activation in cells inside or outside of the immune system (Crabtree et al., 2002). Recent studies have demonstrated that the vital role of NFAT signalling is indeed not restricted to the immune system. NFAT proteins participate in the regulation of genes influencing

the development and differentiation of numerous mammalian cells and tissues (Horsley and Pavlath, 2002; Pavlath and Horsley, 2003). It has been shown, for instance, that NFAT proteins control multiple steps in myogenesis, chondrocyte differentiation and the development of the cardiovascular system (Chang et al., 2004; Friday et al., 2000; Graef et al., 2001; Graef et al., 2003). In addition, recent evidence suggests that NFAT proteins, and in particular NFATc1, regulate important cellular processes such as proliferation and apoptosis in different cell types including epithelial cells, fibroblasts and preadipocytes (Hogan et al., 2003; Neal et al., 2003; Viola et al., 2005). For instance, growth stimulatory effects of NFATc1 and NFATc2 have been demonstrated in skeletal muscle (Pavlath et al., 2003; Horsley et al., 2003) and heart valve development (Ranger et al., 1998; de la Pompa et al., 1998).

Furthermore, NFAT transcription factors have been shown to control peripheral vascular development during angiogenesis, and to play roles in apoptosis regulation both in immune and nonimmune cells (Graef et al., 2001; Hernandez et al., 2001; Zaichuk et al., 2004). The broad spectrum of NFAT mediated processes and the central importance for growth and differentiation of many different cells and tissues strongly implicates a distinct oncogenic potential of NFAT transcription factors. Of special interest in this context is the role of Ca2+/calcineurin/NFAT signalling in cell cycle control, since calcium signalling has been shown to promote cell cycle progression and G1/S phase transition in a variety of normal cells as well as transformed cells (Lipskaia et al., 2004; Buchholz et al., 2006).

Moreover, it has been demonstrated that overexpression of a constitutively active NFATc1 mutant is sufficient to induce a transformed phenotype in preadipocyte 3T3-L1 fibroblasts, associated with altered expression of cell-cycle related genes such as cyclin D1, cyclin D2 and pRB (Neal et al., 2003). NFATs are also implicated in the induction and progression of haematological malignancies. Active nuclear

NFATc1 is found in cases of Burkitt's lymphoma, diffuse large B cell lymphoma and aggressive T cell lymphoma. In experimental settings of T cell acute lymphoblastic leukaemia (T-All), NFAT activation is calcineurin-dependent and pharmacological inhibition of calcineurin reverses cell growth and induces apoptosis (Mancini et al., 2009). Consistent with these findings, the inhibition of calcineurin causes disease regression in mouse models of leukemia (Medyouf et al., 2008).

Our laboratory have recently demonstrated that NFATc1 and NFATc2 are ectopically expressed and highly activated in pancreatic cancer cells in vivo and in vitro (Koenig et al., 2009). In immunohistochemical analyses, more than 85% of human pancreatic cancer specimens and all of the seven pancreatic cancer cell lines analyzed displayed strong nuclear staining for at least one of the two members. We now describe a critical function of NFATc1 and NFATc2 transcription factors in pancreatic carcinogenesis and demonstrate that the induction and activation of both proteins is essential for TGF-β to switch from a growth suppressor to an inducer of cancer cell proliferation. TGF-β induces in a calcineurin dependent manner the transcription of both proteins, which in turn function as downstream effectors to induce c-Myc transcription and cell cycle progression in cancer cells. Site directed mutagenesis combined with overexpression and knockdown studies revealed that NFAT proteins accumulate in the nucleus upon TGF-β stimulation and then target the c-Myc promoter to induce its expression. NFAT factors bind to the c-Myc/TIE element and displace Smad3 repressor complexes from the promoter, as evidenced by chromatin immunoprecipitation assays and reporter gene experiments, and this is ultimately linked with a strong induction of c-Myc expression in cancer.

Thus, these findings have several important implications. First our results support the idea that pancreatic cancer cells can lose their ability to respond to TGF-β with growth inhibition despite the lack of inactivating Smad mutations. Second, we

show that TGF-β can signal simultaneously through different and opposing sets of intracellular pathway, e.g. the NFAT and the Smad pathway. In fact, Smad repressor complexes are still operative in cancer cells without inactivating mutations of the pathway, and thus can act on the c-Myc promoter to silence gene expression. However, stimulation with TGF-β induces activation of the pro-proliferative NFAT cascade and these factors terminate Smad mediated repression through displacement from the promoter. Third, these results strongly suggest that the growth promoting properties of TGF-β are Smad independent and require sufficient activation of non-Smad signalling pathways, such as the calcium responsive NFAT factors.

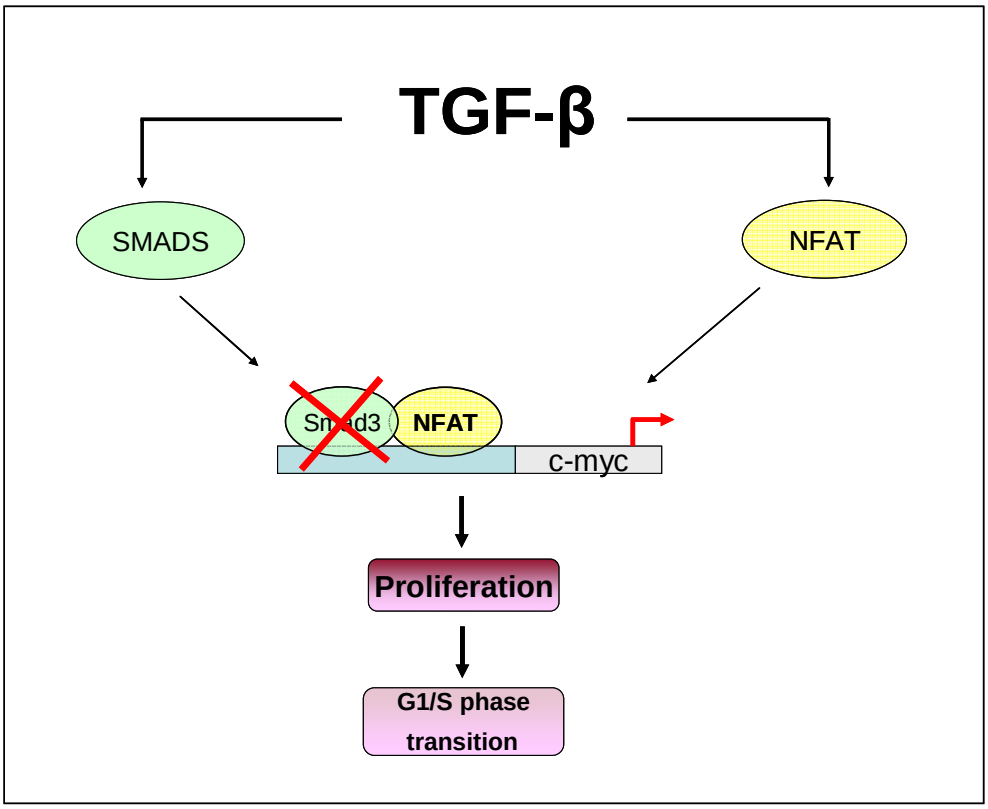

Figure 31. Diagrmatic representation of TGF-β mediated growth promotion in cancer cells. Diagram showing TGF-β mediated pancreatic cancer cell proliferation via NFAT dominancy on c-Myc promoter by antagonizing Smad3 trascription factor.

5.1 SIGNIFICANCE OF MY WORK:

Work over the last years has considerably increased the knowledge about the molecular mechanisms implicated in the TGF-β signal transduction pathway. At a first glance, the TGF-β pathway seems quite linear, but it is turning to be complex, finely regulated and integrated with other signalling pathways. Due to its strong anti-proliferative and apoptotic effects on epithelial cells, TGF-β is considered a tumor suppressor and this function is clearly dependent on the Smad-signalling cascade (Chen et al., 2002). During cancer progression, tumor cells evade from TGF-β action either by acquiring mutations in components of the TGF-β pathway or by selectively inhibiting its anti-proliferative response (Massague et al., 2008). In the latter case, TGF-β not only loses its anti-tumoral function but can also become an oncogenic factor inducing proliferation, angiogenesis, invasion, metastasis and immune suppression (Seoane et al., 2006).

This is the first report demonstrating that TGF-β directly induces the promoter of NFAT transcription factors, leading to increased expression and activation, and results in nuclear accumulation. Even more importantly, following activation NFAT factors bind to and stimulate transcription from the proximal c-Myc promoter and this is a key event in stimulation of G1/S phase cell cycle transition in cancer. The most important finding, however, is that NFAT operate through the same promoter sequence that has been identified previously as the core region in c-Myc promoter silencing. Mechanistically, NFAT must displace Smad3 repressor complexes from the proximal c-Myc promoter to bind and transactivate transcription.

Together, this work significantly contributes to a better understanding of TGF-β growth promotion and the underlying transcriptional mechanisms. Moreover, we identified NFAT factors as novel TGF-β inducible and Smad-independent

transcription factors that work downstream and compete with Smads on target promoters to drive the expression of genes important for cell cycle stimulation. Finally, our data also provide a platform for the development of novel strategies in pancreatic cancer therapy which are based on specific targeting of NFAT factors. Ongoing animal studies in our laboratory will show whether NFAT inhibition is an attractive concept in a modern therapeutic setting to defend pancreatic cancer.

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7 ABBREVIATIONS

AP1	Activating protein-1
APS	Ammonium per sulfate
ATF-3	Activating transcription factor-3
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CBP	CREB-binding protein
CdK	Cyclin- dependent kinase
ChIP	Chromatin Immunoprecipiation
CK-1	Casein kinase-1
Co-Smad	Common- mediator Smad
CsA	Cyclosporin A
DMEM	Dulbecco´s Modified Egle Medium
DNA	Deoxyribonucleic acid
Dnase	Deoxyribonuclease
DPC4	Deleted in Pancreatic Cancer, locus 4
DTT	Dithio-threitol
E-Box	Enhancer box sequence
E.Coli	Escherichia coli
EDTA	Ethylene-diamine-tetra-acetic acid
EMT	Epithelial mesenchymal transition
ERK	Extra cellular signal regulated kinase
Evi-1	Ecotropic viral integration site 1
E2F	Electro-acoustic 2 factor
FCS	Fetal calf serum
Fox	Forkhead box

G1	Gap1
HCL	Hydrochloric acid
HDAC	Histone deacetylase
HEPES	Hydroxyethyl-piperazineethanesulfonic acid
HRP	Horse readish peroxidase
Id-1	inhibitor of differentiation-1
IgG	Immunoglobulin G
IkB	Inhibitor of NFkB
I-Smad	Inhibitory Smad
JNK	Jun-kinase
Kd	Kilo dalton
KLF	Kruppel-like factor
LAP	Latency associated peptide
LB-Medium	Luria-Bertani-medium
LiCl	Lethium chloride
Mad1	Max dimerization protein-1
MgCl2	Magnesium chloride
MH	MAD homology
Miz-1	Msx-interacting zing finger -1
mSin3	Mammalian Sin3
MAPK	Mitigen activated protein kinase
MMP	Matrix metalloprotease
MSI	Microsatellite instability
Myc	v-myc myelocytomatosis viral oncogene
NaCl	Sodium chloride
Ncor	Nuclear receptor co-repressor 1
NES	Nuclear export signal
NFAT	Nuclear factor of activated T cell
NLS	Nuclear localization signal

PAI-I	plasminogen activator inhibitor-1
PCR	Polymerase Chain Reaction
PI3K	Phospho-Inostiol-3kinase
PMSF	Phenylmethylsulfonylfluorid
Rb	Retinoblastoma protein
RLU	Relative luciferase unit
RNA	Ribonucleic acid
R-Smad	Receptor- Smad
RT	Room temperature
RUNX	Runt-related transcription factor X
S-phase	Synthesis phase
SARA	Smad anchor for receptor activation
SBE	Smad binding element
SDS	Sodiumdodecylsulfate
SID	mSin3A-interacting domain
Ski	Sloan- Kettering Institute proto oncogene
SnoN	Ski-related novel gene N
Sp	Specificity protein
TEMED	N, N, N', N'-Tetramethylethylenediamine
TF	Transcription factor
TFE	Transcription factor enhancer
TGIF	TG3-inetrating factor
TIE	TGF-β inhibitory element
TβRI	TGF-β type I receptor
TβRII	TGF-β type II receptor
TGIF	TG-3- interacting factor

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G. Singh, SK Singh, A. König, K. Reutlinger, TM Gress and V. Ellenrieder. NFAT mediate TGF-β switch from a growth suppressor to a promoter of cell proliferation. Accepted for poster presentation in *"Transcription and Cancer meeting"* held at Churchill College Cambridge", UK from 8th - 10th July 2009.

G. Singh, S.K. Singh, A. König, K. Reutlinger, T.M. Gress, Martin E. Fernandez-zapico and V. Ellenrieder. NFAT and c-Myc at the core of TGF-β induced pancreatic cancer cell growth. Accepted for poster presentation in *"39th Annual meeting of American pancreatic association"* Chicago, USA from 3rd - 7th November 2008.

SK Singh, **G. Singh**, A. König, K. Reutlinger, G. Suske, LC Hofbauer, TM Gress and V. Ellenrieder. The Antitumor Effects of Zoledronic acid Are Mediated by HDM2 Induced Proteasomal Degradation Of Oncogenic NFATc2. Accepted for poster presentation in *"2nd Salk Institute Mechanisms & Model of Cancer meeting"* held at Salk Institute for Biological Studies, La Jolla, CA, USA from 12th - 16th August 2009.

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Declaration

I hereby declare that the submitted dissertation was completed by myself and none other and I have not used any sources or materials other than those enclosed. Moreover, I declare that the following dissertation has not been submitted further in this form and has not been used for obtaining any other equivalent qualification in any other organization. Additionally, other than this degree I have not applied or will not attempt to apply for any other degree, title or qualification in relation to this work.

Garima Singh

Date 15.04.2010

Marburg

Ehrenwörtliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin Marburg zur Promotionsprüfung eingereichte Arbeit mit dem Titel **„Molecular characterization of TGF-β mediated cancer cell proliferation“** in der Klinik für Innere Medizin unter Leitung von Herrn Prof. Dr. Thomas Mathias Gress mit Unterstützung durch Prof. Dr. Volker Ellenrieder ohne sonstige Hilfe selbst durchgeführt habe. Bei der Abfassung der Arbeit habe ich keine anderen als die in der Dissertation angeführten Quellen und Hilfsmittel benutzt und vollständig oder sinngemäß übernommene Zitate entsprechend gekennzeichnet. Ich habe bisher weder an einem in- und ausländischen Medizinischen Fachbereich ein Gesuch um Zulassung zur Promotion eingereicht noch eine andere Arbeit als Dissertation oder die vorliegende zu anderen Prüfungszwecken vorgelegt.

Garima Singh

Date: 15.04.2010

Marburg